IN VIVO EFFECTS OF PEROXOVANADIUM (PV) COMPOUNDS AS HYPOGLYCAEMIC AGENTS

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Abstract

Vanadium is a trace element found in most living systems. It has various biological properties. In the last 10 years numerous reports showed that Vanadium administered orally could improve the metabolic state of diabetic animals. The combination of vanadate and H_2O_2 produced the new agents called peroxoVanadium (pV) compounds which exerted more potent insulin mimetic effects by activating the insulin receptor kinase. Their application in the treatment of diabetes could be of great benefit clinically in the future. We demonstrated that long-term oral bpV (phen) (a new pV compound) treatment resulted in a lowering of blood glucose levels, with less toxicity than vanadate in STZ-diabetic rats. bpV(phen) was the first agent other than insulin, that was able to maintain the insulin-deprived diabetic BB rats in good apparent health without ketonuria for 25 days by intraperitoneal injection (IP). Although bpV(phen) was ineffective in reducing glucose levels by IP injection in BB rats, it caused a significant decrease of insulin and C peptide levels and in the insulin dose required to maintain the aglycosuric state of the diabetic BB rats. These observations are consistent with a mechanism of bpV(phen) action at tissue levels. The exploration of the properties of pV compounds may help elucidate both the mechanisms of insulin action and the cause of diabetes, and also may give rise to insulin substitutes which could be orally administered for the treatment of diabetes in the future. However IP injection of bpV (phen) also caused severe toxic side effects. The toxic effects of bpV(phen) included inhibition of food and water intake, acceleration of the onset of diabetes and death. Further studies are required to identify second generation pV compounds with better therapeutic/toxicity ratios and to find ways of delivering pVs orally.

Résumé

Le vanadium est un élément trace retrouvé dans la plupart des systèmes vivants. Il est impliqué dans plusieurs processus biologiques. Au cours des 10 dernières années, il fut décrit que le vanadium oral améliorerait l'état métabolique d'animaux diabétiques. La combinaison de vanadate et d'H_2O_2 a produit de nouveaux agents appelés composés de pV qui possèdent une activité insulino-mimétique plus puissante en activant la kinase du récepteur à l'insuline. Nous avons démontré qu'une thérapie orale à long terme avec du bpV(phen) diminuait la glycémie de rats diabétiques-STZ avec moins de toxicité que le vanadate. Le bpV(phen) administré par voie intrapéritonéale (IP) fut le premier agent, autre que l'insuline, à pouvoir maintenir des rats BB diabétiques en bonne santé apparente sans cétonurie pendant 25 jours. Bien que le bpV(phen) injecté IP fut incapable de réduire la glycémie des rats BB, il causa une diminution significative des niveaux d'insuline et de peptide C circulants chez les rats BB non-diabétiques, et des doses d'insuline requises pour maintenir l'état non-glucosurique des rats BB diabétiques. Ces observation supportent l'idée que le bpV(phen) agit au niveau des tissus cibles. L'exploration des propriétés des composés de pV pourrait aider à élucider les mécanismes d'action de l'insuline et la cause du diabète, et pourrait mener à de nouveaux substituts de l'insuline qui pourraient être administrés par voie orale dans le futur. Cependant, l'injection IP du bpV(phen) mène à de nombreux effets toxiques, incluant une diminution de l'ingesta de nourriture et d'eau, l'accélération du diabète et la mort. Des études supplémentaires seront nécessaires pour mener à des composés de pV de seconde génération avec de meilleurs rapports bénéfices/toxicité et pour trouver des méthodes d'administration orale efficaces.

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CHAPTER 1: INTRODUCTION

Diabetes

Historical Background

Diabetes was recognized more than 2000 years ago. The symptoms of increased appetite, thirst and a large volume of urine had been described as a disease in ancient Chinese medical records. A Greek doctor, Aretaeus (A.D. 70), described the condition and gave it its name: Diabetes. In the sixteenth century, Paracelsus recognized the sweetness of diabetic urine. In 1869, Langherhans described the pancreatic endocrine cells situated in the islets which bear his name. In 1889, Minkowski and Von Mering showed that removal of the pancreas in dogs led to diabetes. The discovery of insulin at the University of Toronto in 1921 was one of the most dramatic events in the history of treatment and diagnosis of diabetes. In 1922, the first patients were treated with this life saving hormone - insulin in the Toronto General Hospital (1,2).

What is Diabetes?

Diabetes mellitus, a syndrome characterized by chronic hyperglycaemia, is a disorder of carbohydrate, fat, and protein metabolism associated with absolute or relative insufficiency of insulin secretion and with various degrees of insulin resistance.

When diabetes is fully expressed, it is characterized by fasting hyperglycaemia, but the disease can also be recognized in the early stage by the presence of glucose intolerance. Excessive thirst, polyuria, unexplained weight loss and chronic fatigue are the characteristic symptoms of diabetes mellitus (3,4).

Classification of diabetes:

Although all those with diabetes lack effective insulin action, differences between various forms of the disease are expressed in terms of cause and pathogenesis, environmental, and immune factors, natural history, and response to treatment. The most common types of diabetes are Type I and Type II.

Type I diabetes is characterized by the acute appearance at a young age of hyperglycemia, glycosuria and weight loss, leading to rapid death unless insulin therapy is instituted. Insulinopenia and islet cell antibodies are present. Evidence regarding etiology suggests genetic and environmental or acquired factors, association with certain HLA types, and abnormal immune responses, including autoimmune reactions.

Type II diabetes is characterized by insulin resistance and obesity. People affected by this disease are not

insulin-dependent or ketosis-prone, although they may use insulin for correction of symptomatic or persistent hyperglycemia. Serum insulin levels may be normal, elevated, or depressed. Onset is usually after age 40. Insulin resistance and obesity are usually present (3,5).

Pathophysiology of diabetes mellitus:

Most of the pathologic features of diabetes mellitus can be attributed to one of the following three major effects of the lack of insulin: (1) decreased utilization of glucose by the body cells, resulting in elevated blood glucose concentrations to as high as 15 to 60 mmol/L; (2) markedly increased mobilization of fats from fat storage areas, causing abnormal fat metabolism as well as deposition of cholesterol in arterial walls, causing atherosclerosis; and (3) depletion of protein in the tissues of the body(6).

Effects of Elevated Blood Glucose levels in diabetes:

The hyperglycaemia increases serum osmolarity. The increased osmotic pressure in the extracellular fluids causes osmotic transfer of water out of the cells. When the amount of glucose entering the kidney tubules rise above a critical level, the "glucose threshold", the excess glucose then cannot be absorbed and spills into the urine. The loss of glucose in the urine causes osmotic

diuresis. Thus, one of the important features of diabetes is extracellular and intracellular dehydration which can contribute to the development of circulatory shock (6).

Abnormal fat metabolism:

Diabetic ketoacidosis is an acute complication that is fatal if not treated. When carbohydrates are not utilized for energy, almost all the energy of the body must come from metabolism of fats. When the body depends almost entirely on fat for energy, ketone body formation is greatly increased, which results in acidosis. Ketone-bodies are synthesized in the liver from fatty acids. Fatty acids are taken up by the liver and synthesized in the cell cytosol to triglyceride or alternatively they may enter the mitochondria as fatty acyl CoA. Inside the mitochondria, fatty acyl CoA undergoes β oxidation to acetyl CoA, which is then either totally oxidized in the Krebs' cycle or converted into acetoacetic acid, β-OH butyrate, and acetone together called ketone bodies. The acetoacetic acid and β-OH butyrate are transported by blood to the peripheral tissues, where they reverse to acetyl-CoA and are oxidized for energy. One of the products of carbohydrate metabolism is oxaloacetate that is required to combine with acetyl-coA before it can be processed as citrate in the Kreb's cycle. Therefore, deficiency of oxaloacetate limits the entry of acetyl-CoA

into the Kreb's cycles, and simultaneously helps promote production of acetoacetic acid and ketone bodies. Insulin increases the production of malonyl CoA which is an inhibitor of carnitine palmitoyl transferase-1 (CPT1). By contrast, glucagon stimulates CPT1 by decreasing malonyl CoA. CPT1 is the rate-limiting enzyme allowing further metabolism of fatty acid to ketone bodies in the mitochondria. Therefore, the reduced inhibition to CPT-1 as in insulin deficiency, increases the formation of the ketone bodies. However, the absence of insulin also depresses the utilization of acetoacetic acid and β-OH butyrate in peripheral tissues. All these extra acids cannot be metabolized by the tissues and result in acidosis which can lead to acidotic coma and death within hours when the pH of the blood falls below about 7.0 without proper treatment (7,8).

Macrovascular complications of diabetes represent one of the most serious consequences of the disease. Almost all blood vessels in the body, both large and small, are affected in patients with long standing diabetes. A major factor that contributes to macrovascular damage appears to be abnormal lipid metabolism. In the absence of insulin the enzyme hormone-sensitive lipase in the fat cells becomes strongly activated. This causes hydrolysis of the stored triglycerides releasing large quantities of fatty

acids and glycerol into the circulating blood. Following uptake by the liver there is increased production of low density lipoproteins and triglycerides which could accelerate the atherosclerotic process leading to macrovascular complications (9,10).

Alterations in protein metabolism in diabetes mellitus: Untreated diabetes mellitus is accompanied by severe muscle wasting resulting from increased protein catabolism. Insulin is an anabolic hormone, and its presence in the blood promotes the storage of protein. Insulin increases the uptake of amino acids into tissues, including muscle, liver, and adipose tissue, and stimulates protein synthesis and inhibits protein degradation. Under diabetic conditions, in which the blood level of insulin is depressed, the protein stores, particularly those in muscle are reduced. Amino acids released from protein are used as fuel. Alanine and glutamine released into the blood become substrates for gluconeogenesis in the liver and kidney respectively. Under this condition, whole-body proteolysis is significantly increased, and causes severe muscle wasting. Treatment with insulin can correct the defect in protein catabolism (11,12).

Diagnostic Criteria for Diabetes and Impaired Glucose

Tolerance:

The normal level of fasting plasma glucose is below 6.7 mmol/l. A value of 7.8mmol/l or greater in plasma twice establishes the diagnosis of diabetes. A level between 6.7 and 7.8mmol/l in plasma requires a glucose tolerance test to confirm diagnosis.

Treatment of diabetes:

Among the variety of antihyperglycemic agents available, insulin injections remain the major option in type I diabetes and are frequently used in those with type II diabetes. The blood glucose level is mainly affected by plasma insulin levels, by the amount of carbohydrate consumed in the previous three to four hours, and by the amount of exercise and stress. Therefore, the management of people with diabetes includes not only medication but also the regulation of diet, exercise and emotion (13).

Prevention of diabetes:

Recently it was suggested that while inhibiting endogenous insulin secretion helps prevent type I diabetes (15,16). The possible mechanisms include a protection from the immune injury by inhibiting β cell secretion. This may be related to a lower expression of a target antigen because the β cell is at rest. However, the resistance to cellular cytotoxicity of resting beta cells suggests resting beta

cells may also be more resistant to cytotoxic attacks. Since peroxovanadium compounds have powerful insulin-mimetic effects and inhibited endogenous insulin secretion (17), we assessed whether bpV(phen) would be able to reduce the incidence of diabetes.

Insulin

Insulin was first isolated from the pancreas in 1922 by Banting, Best, Collip and Macleod. It soon became the major therapy for all patients with Type I diabetes and for many of those with Type II diabetes (1).

The secretion of insulin from pancreas is mainly controlled by the blood glucose concentration. In turn, insulin plays a key role in controlling blood glucose, fat and protein metabolism. The cellular effects of insulin are very broad and include modulation of: (1) transport of molecules across the plasma membrane; (2) decreased levels of cyclic nucleotides; (3) activities of enzymes in intermediary metabolism; (4) rates of protein synthesis and degradation; (5) rate of DNA and RNA synthesis, including specific gene expression; and (6) cellular growth and differentiation (18,19).

Insulin structure :

Insulin is a small protein with a molecular weight around 5900 Kd. It is composed of two amino acid chains, connected to each other by disulfides linkages. When the two amino acid chains are split apart, the activity of the insulin molecule is lost. Insulin has a compact three dimensional structure (19).

Secretion and metabolism of insulin, proinsulin and C-peptide:

Insulin is synthesized in the β-cells of pancreas by the usual cell machinery for protein synthesis, beginning with translation of the insulin mRNA on ribosomes attached to the endoplasmic reticulum to form an insulin preprohormone. This initial preprohormone has a molecular weight of about 11,500 Kd, and is then cleaved in the endoplasmic reticulum to form proinsulin with a molecular weight of about 9000 Kd. Proinsulin is the immediate biosynthetic precursor of insulin and is converted in the β-cell's secretory granules to one molecule of C-peptide and one molecule of insulin, each of which is secreted into the portal circulation. However, about one sixth of the final secreted product is still in the form of proinsulin, which has little (about 10% on a molar basis) insulin activity.

The role of the C-peptide relates largely to maintaining

the tertiary structure of proinsulin during insulin biosynthesis. It does not possess independent biological activity after secretion into the circulation. C-peptide has been extremely useful as a marker of insulin secretion, since it is co-secreted in equimolar concentration with insulin. Unlike insulin, it does not undergo significant hepatic extraction and is excreted almost exclusively by the kidneys. Its plasma half-life is approximately 30 minutes (20,21).

The Clearance of Insulin:

When insulin is secreted into the blood, it circulates almost entirely in an unbound form: It has a plasma half-life averaging about 6 minutes, so that it is largely cleared from the circulation within 10 to 15 minutes. Except for that portion of insulin combined with receptors in target cells (22).

Regulation of Secretion:

Plasma glucose is the most important regulator of insulin release. The effect of glucose on the beta cell is dose-related. Insulin secretion does not respond as a linear function of glucose concentration. The β-cell insulin secretory response is greater following oral than intravenous glucose administration(21).

Although glucose is the major regulator of insulin secretion by pancreatic beta cells, its action is modulated by several neural and hormonal stimuli. In particular, hormones secreted by intestinal endocrine cells stimulate glucose-induced insulin secretion very potently afternutrient absorption. These hormones, called gluco-incretins or insulinotroption hormones, are major regulators of postprandial glucose homeostasis. The main gluco-incretins are GIP(gastric inhibitory polypeptide-1) and GLP-1 (glucagon like peptide 1)(23).

Effect of Insulin on Metabolism:

Insulin plays a critical role in building and breaking down energy reservoirs in humans. After a meal, insulin levels increase, promoting glycogen synthesis in liver and muscle, lipid formation in adipocyte, and amino acid uptake and protein synthesis in most cells. Between meals, during starvation and in response to many stresses, decreased insulin levels result in glycogen breakdown, lipolysis, hepatic ketogenesis, and decreased synthesis and increased degradation of protein (24).

The Mechanism of Insulin Control over Glucose Uptake, Storage and Release:

Insulin plays a key role in the regulation of glucose

metabolism. First of all, insulin increases cell permeability to glucose up to 10 to 20-fold by fusion of multiple intracellular vesicles with the cell membrane. These vesicles carry multiple molecules of glucose transport protein (24).

In liver cells, insulin increases the activity of the enzyme glucokinase which increases the rate of phosphorylation of glucose in liver cells. Once phosphorylated, the glucose is temporarily trapped inside live cells, because phosphorylated glucose cannot diffuse back through the cell membrane. Insulin also increases the activities of a number of enzymes including phosphofructokinase, which catalyses the second stage in the phosphorylation of the glucose molecule; and glycogen synthetase, which is responsible for polymerization of the monosaccharide units to form glycogen. In skeletal muscle, it causes an activation of protein phosphatase-1 via phosphorylation on serine/threonine residues, which stimulates the dephosphorylation of glycogen synthase and phosphorylase kinase leading to inhibition of glycogenolysis and an activation of glycogen synthesis. The decrements in the plasma insulin concentration between meals reverses all the effects mentioned above for glycogen storage, essentially stopping further synthesis of glycogen in the liver and preventing further uptake of

glucose by the liver from the blood. In addition, the decline of inhibition of phosphorylase and glucose-6 phosphatase by lower insulin concentrations causes the splitting of glycogen into glucose allowing the free glucose to diffuse back into the blood (24).

Effects of Insulin on Fat Metabolism:

Insulin is a powerful inhibitor of lipolysis. The glucose-induced rise in plasma insulin concentrations quickly inhibits lipolysis by inhibiting hormone-sensitive lipase in the adipocyte. This reduces the supply of lipid substrates to the oxidative machinery in muscle and liver. In addition to its potent restraining effect on lipolysis and hence inhibition of FFA oxidation, insulin stimulates fatty acid synthesis and storage as triacylglycerol in adipose depots throughout the body. Insulin augments fatty acid synthesis in adipose tissue and the liver primarily by activating acetyl-CoA carboxylase. Insulin promotes glucose transport through the cell membrane into fat cells. Some of this glucose is then utilized to synthesize small amounts of fatty acids; but, more important, it also stimulates the formation of α-glycerophosphate which supplies the glycerol that combines with fatty acids to form the triglycerides in adipose cells (24,25).

Effects of Insulin on Protein Metabolism:

Insulin is not only required for storage of glucose and fat, but also protein. Insulin activates amino acids transporters on cell membrane(especially for valine, leucine, isoleucine, tyrosine and phenylalanine). Insulin stimulates protein synthesis by a direct effect on the ribosomes in increasing the translation of mRNA; and by an effect on transcription of DNA in the cell nuclei. Insulin also suppresses proteolysis thus decreasing the rate of amino acid release from the cells, especially from the muscle cells (24,10).

Insulin Receptor

Insulin initiates action by binding to a cell-surface receptor. The insulin receptor is a glycoprotein on the plasma membrane of almost all mammalian cells. The number of receptors varies among tissues from less than 100 per cell to more than 100, 000 per cell, with the higher concentrations being found primarily on adipocytes and hepatocytes. Insulin binding to its receptor is rapid, reversible, saturable, and has a specificity consistent with the biological potency of insulin and insulin analogues. There are no known competitive antagonists at the receptor level; i.e., all insulin analogues that bind to the receptor exert a biological effect in proportion to their affinity for the receptor (26).

The insulin receptor is an integral membrane glycoprotein consisting of two α (135 kD) and two β (95 kD) chains joined by three disulfide bonds. The α-subunits are completely extracellular and contain a characteristic cysteine rich region, which is critical to the ligand-binding function. In contrast, the β-subunits are composed of an extracellular domain, a membrane-spanning domain and an amino acid intracellular tail that contains an insulin regulated tyrosine protein kinase activity (27).

α - subunit

Insulin action is initiated when insulin binds to the α-subunit of the insulin receptor. Binding occurs with high affinity. High-affinity insulin binding requires amino acid residues in several domains of the α-subunit, including the amino-terminal cysteine-rich region and the carboxy terminus. After insulin binding, the receptor changes its structure, causing phosphorylation of the β-subunit (27).

β - subunit

The extracellular β-subunit region participates in transmitting the insulin signal through the membrane to the cytoplasmic region of the β-subunit.

The 23-amino acid transmembrane region of the β-subunit

anchors the receptor in the plasma membrane. Transmembrane swapping experiments and mutagenesis studies suggest that, other than anchoring the receptor in the membrane, the specific sequences of the insulin-receptor transmembrane domain are not essential for signaling function (27).

The cytoplasmic portion of the β-subunit contains a tyrosine kinase domain, and several autophosphorylation sites in insulin-receptor-specific sequences. When insulin binds to the α-subunit, the tyrosine kinase property of the β-subunit is stimulated, allowing the receptor to autophosphorylate rapidly on specific tyrosine residues and to become an active tyrosine kinase which in turn causes phosphorylation of multiple other intracellular enzymes. In this way, insulin accomplishes its function. From this point on, the molecular mechanisms are not well known, but two potential mechanisms have been considered as the most likely involved in signal transduction: 1) a phosphorylation cascade initiated by the active tyrosine kinase of the receptor; and 2) the generation of a second messenger (27).

<u>The complications of insulin treatment:</u>

Currently, insulin is still an essential treatment for type I diabetes. Insulin is the only agent to eliminate hyperglycemia and prevent ketoacidosis and other

complications. However, insulin cannot be given in a manner that perfectly duplicates the normal patterns of insulin in the circulation as it is secreted from the islets in the postprandial and basal states. Insulin treatment causes many problems, by far the most frequent problem in patients treated with insulin for diabetes is hypoglycemia. Severe hypoglycemia can cause loss of consciousness, brain damage and death, and fear of developing hypoglycemia can affect patient behaviour and approach to insulin treatment. Insulin antibodies, insulin allergy, insulin resistance and weight gain can also occur. Because oral administration of insulin is ineffective, insulin must be injected. Multiple injections cause problems in patients, particularly in those who are reluctant or unable to administer insulin injections to themselves several times a day for the rest of their lives (28).

Vanadate

Vanadate is an essential nutrient for normal mammalian development and various biological processes. Vanadium was first discovered in 1831. Because of the rich colours of the vanadium crystal and solutions, the element was named after Vanadis, the Scandinavian goddess of youth and beauty (29,30).

Many vanadium-dependent physiological and biochemical effects have been established. It was found that vanadium was an essential nutrient for optimal growth of chicks and rats, and a potent mitogen for several cell types. Vanadium also altered a variety of enzyme activities, including myosin ATPase, adenyl cyclase and interacts synergistically with growth factor such as EGF, and IGF-II (30,31)

Interest has been growing particularly regarding its antidiabetic effects. The first suggestion that vanadium compounds could influence glucose homeostasis was probably reported by Lyonnet, et al. in 1899, when they found that oral vanadium decreased glucosuria (29). Since then, more and more evidence that vanadium improves glucose homeostasis has been found (29).

<u>In vitro effects of vanadium:</u>

In the past ten years, extensive studies have been performed examining the mechanism through which vanadium mimics the action of insulin and prevents the signs of diabetes. Initially researchers supported the mechanism that vanadium is a potent phosphotyrosyl phosphatase inhibitor (32). Schecter et al.(1993)argued that vanadate acts via a cytosolic tyrosine kinase (33).

The insulin-mimetic action of vanadium has been shown in various cell types. Vanadium stimulated glucose transport, oxidation and glycogen synthesis and inhibited gluconeogenesis in adipocytes, skeletal muscle and hepatocytes (34,35,36). In the liver, vanadium regulated glucose metabolism via a variety of enzymes including the activation of glycogen synthase and the inhibition of fructose-2, 6-bisphosphatase and glucose-6-phosphatase. Vanadium has also been shown to restore the expression of genes for PEPCK and GLUT-2 which are key enzymes in glucose and ketone body metabolism in the liver of rats (33,29). In addition to its effects on glucose, vanadium like insulin, also regulated fat (and protein) metabolism. In adipocytes, vanadium increased lipogenesis and inhibited lipolysis and protein degradation (34,37).

In vivo effects of vanadium:

The antidiabetic properties of orally administered vanadium have been well documented. Heyliger, et al. (1985) first reported that sodium orthovanadate, supplied in the drinking water of streptozotocin-diabetic rats, lowered blood glucose levels and reversed cardiac abnormalities, without increasing insulin levels (29,38). Subsequent studies demonstrated that orally administered vanadium decreased blood glucose levels within 2-4 days after treatment with high concentration (0.8 mg/ml in

drinking water), and reduced blood glucose within 4 days at a lower concentration (0.2mg/ml)(39). Pederson, et al. indicated that blood glucose levels in STZ-diabetic rats normalized after three weeks of vanadyl treatment and remained normal up to 13 weeks after vanadyl treatment withdrawal(40). Higher insulin levels were found in most vanadium studies in Streptozotocin-diabetic rats (41). The higher insulin levels suggested that the STZ-diabetic rats did have significant residual insulin which might have been secreted from regenerated β-cells (41). The efficiency of vanadyl treatment was however unrelated to protection from the acute toxic effects of streptozotocin on pancreatic β cells (38).

Toxicity:

The studies described above suggest that vanadium is a potential agent for future diabetes treatment. It is therefore critical to understand the possible side-effects associated with vanadium therapy before supplying it to humans.

The overall toxicity of vanadium depends on the route of administration. The oral route appears to be much safer than the intraperitoneal and intravenous routes probably because vanadium is only poorly absorbed by the intestine. Though the oral route was safer than other routes, the

doses of vanadium used orally were still higher than those considered to be toxic in normal rats. Commonly described signs of vanadium toxicity in experimental animals include diminished food and water consumption, dehydration, diarrhea, weight loss, death and increased serum concentrations of urea and creatinine (42,45,47).

Vanadium ingestion might induced water and food retention which subsequently was responsible for the diminished water and food intake (42,45,47). The reduction in body weight gain appeared to be due to a decrease in food consumption (41). It was shown that the anorexic effect of vanadium was due to the central nervous system (29,34). It was reported that vanadate did not inhibit feeding at the hypothalamic level, because vanadate did not directly inhibit neuropeptide Y (NPY) synthesis and transport (34).

In addition, toxic effects of vanadium on red blood cells of rats was reported. It was found that vanadium may affect maturation of red blood cells (44).

Furthermore, J.L Domingo, et al. reported that vanadium accumulated in kidneys, bone, and spleen following treatment with vanadyl for a period of 3 months. The extent of tissue accumulation of vanadium in diabetic animals might imply an additional risk to several cell

types, considering vanadate's capacity to alter various enzyme activities (42,45,47). Vanadate were described to decrease the myocardial contractile force, probably due to a large intracellular accumulation of calcium and cell damage (48).

Little is known about the possible toxic effects of vanadium in humans. Following exposure by the respiratory route in industrial settings, chronic toxicity included gastro-intestinal distress, fatigue, green tongue, palpitations and damage to the kidneys, liver and nervous system were described (30,31).

The toxicity of vanadium also depends on the chemical form of vanadium and the element coadministered. It was found that vanadyl was less toxic than vanadate in alleviating some signs of diabetes in STZ-diabetic rats (29). The vanadyl rather than vanadate form may be more appropriate for chronic use in diabetic rats. Tiron has remarkable efficacy in mobilizing vanadium, and coadministration of Tiron to vanadate-treated diabetic rats may result in reduced accumulation of vanadium in liver, kidneys, heart, bone and muscle (47). Coadministration with NaCl (0.8mg/ml NaCl) has also been shown to reduce vanadium toxicity.

Peroxovanadium (pV) compounds:

Posner, et al. (1987) found that the combination of vanadate and H_2O_2 produced a powerful synergistic effect in mimicking insulin and activating the insulin receptor kinase (IRK) in rat adipocyte. Further studies have shown that the action of pV compounds involves the insulin receptor, and exceeds the individual effects of vanadate or H_2O_2 (50,51). Therefore, pV compounds might be a useful tool for studying the mechanism of insulin action, and a new therapeutic model for the management of diabetes.

The chemistry of pV Compounds:

pV compounds were synthesized, crystallized and characterized by ^{51}V NMR as >95% pure (Posner B.I.,et al.1994). Recently, twelve new pV compounds were synthesized. Each compound contains an oxo ligand, one or two peroxo anions, and an ancillary ligand in the inner coordination sphere of vanadium. The potency of these pV compounds as IRK activators and phosphotyrosyl phosphatase (PTPs) inhibitors in cultured cells and in vitro was influenced by the various ancillary ligands (32).

The mechanism of action of pV compounds:

Posner et al (1994) reported that pV compounds inhibited the activity of an IRK-associated PTPs. The direct and

significant correlation between IRK activating and PTPs inhibiting potencies of the pV complexes and the parallel loss of these two activities during compound breakdown in solution along with a capacity to augment insulin receptor phosphotyrosine content supported the view that they acted by inhibiting IRK dephosphorylation.

In vitro effects of peroxovanadium:

pV compounds had more potent insulin mimetic effects than vanadium both in vitro (32,51,53) and in vivo (17,41). Once the receptor was autophosphorylated and completely activated by arresting dephosphorylation, the insulin receptor could transduce the signals leading to stimulated glucose transport, lipogenesis, antilipolysis and protein synthesis (32). Pervanadate was not only able to mimic insulin in stimulating glucose uptake, but also can significantly enhance the maximum cell responsiveness achieved at saturating insulin concentration in rats adipose cells (33). However, vanadium, when added to saturating concentrations of insulin, did not exceed the rate and extent of glucose uptake achieved at the maximal dose of insulin in rats adipose cells. Peroxovanadate at 0.1 mmol/l was as effective as insulin in inhibiting lipolysis in human adipose cells , whereas no insulin-like effects were exerted by vanadate (50). The potency of pervanadate to stimulate lipogenesis was comparable to

that of insulin and 10^2-10^3 times more potent than vanadate in rats adipose cells. Like insulin pervanadate stimulated protein synthesis up to a maximum of 23% over the control group, whereas vanadate had only minimal or no effect on protein synthesis in rats adipose cells. Higher concentrations of vanadate were found to inhibit protein synthesis (51).

In vivo effects of peroxovanadium:

A significant correlation was found between the capacity of the pV compounds to inhibit PTPs and their in vivo hypoglycemic potency (41). The long duration of the hypoglycemia following treatment with bpV(phen) was consistent with the more extended time course of insulin receptor kinase activation produced by this compound (32).

pV compounds produced much greater and more rapid effects on plasma glucose than vanadium (41). The subcutaneous administration of pV compounds to insulin-deprived BB rats over a 3 day period produced a persistent lowering of both blood glucose and ketone body levels. These hypoglycaemic effects were not due to a decrease in food intake because diabetic BB rats fasting for the same period of time showed persistent hyperglycaemia and increasing ketonuria (41). Assia Shisheva, et al.(1994) reported that a single intraperitoneal injection of pervanadate had a dramatic

effect in improving glucose homeostasis in STZ diabetic-rats at doses corresponding to only 700 μg vanadium/kg body weight (14). The requirement for insulin in diabetic BB rats is reduced when vanadyl is coadministered with insulin, but vanadyl could not replace insulin entirely either with respect to the control of glycaemia or the anabolic effects of insulin (34). The decrease in plasma glucose effected by pV compounds was accompanied by a decrease in circulating insulin levels in Sprague-Dawley rats indicating that the hypoglycemic effect occurred in peripheral insulin target tissues instead of via the augmentation of circulating insulin levels (17). In contrast, most vanadate studies have shown higher plasma insulin levels in STZ-diabetic rats following vanadate treatment (41). The higher insulin level might be related to the regeneration of β-cells (40).

In addition to stronger insulin-mimetic effects pV compounds were less toxic than vanadium when injected parenterally. pV compounds injected intravenously produced a 20 % decrease in plasma glucose without causing mortality, but the intravenous administration of sodium orthovanadate or vanadyl sulfate produced mortality before any significant reduction of plasma glucose level could be observed (41).

CHAPTER 2: HYPOTHESIS AND OBJECTIVE OF THE STUDY

Previous studies have shown that Vanadium can improve the diabetic state and prevent the development of diabetic complications in both insulin-dependent and non-insulin-dependent diabetes mellitus in experimental animals by the oral route of administration (29,38,43). Peroxovanadium compound were much more potent than vanadium as insulin-mimetic agents both in vitro (32,51,53) and in vivo (17,41). Therefore, pV compounds might be useful new agents for the treatment of diabetes.

Diabetes is very common and is a life-long disease. Insulin remains the only available treatment in the management of type I diabetes and is frequently used in those with type II diabetes. However, insulin cannot be given in a manner that perfectly duplicates the normal patterns of insulin in the circulation as it is secreted from the islets in the postprandial and basal states. Insulin treatment also causes many problems,such as hypoglycemia, insulin antibodies, insulin allergy, insulin resistance and weight gain (28).

Oral administration of insulin in mammals is ineffective. Therefore, the availability of orally administered insulin substitutes could be of importance in the treatment of

diabetes. This is particularly valid as: (a) many patients are reluctant or unable to administer insulin injections to themselves several times a day for the rest of their life; and (b) a large variability in insulin absorption occurs in diabetic patients (28).

Besides insulin deficiency, resistance of target tissues to insulin is another major cause of diabetes (1,2). pV compounds which increase tissue sensitivity to insulin or mimic its action may thus be extremely useful for controlling the disorder of glucose metabolism caused by insulin resistance.

Recently, studies have shown that inhibiting insulin secretion helps prevent type I diabetes (54,15). A possible mechanism is through protection from immune injury by inhibiting β cell secretion, and the expression of target antigens. Our previous studies had reported that the decrease in blood glucose effected by pV compounds was accompanied by a decrease in circulating insulin levels (17). These results provide basis for studying whether pV compounds could reduce the development of diabetes by decreasing endogenous insulin secretion.

Therefore, the exploration of the properties of pV

compounds may give rise to insulin substitutes which can be orally administered for the treatment and prevention of diabetes, and also may assist in elucidating both the mechanisms of insulin action and the cause of diabetes in the future.

This thesis is concerned with evaluating the efficiency of different peroxovanadium compounds in diabetic rats (BB Wistar and STZ-diabetes). I performed the studies using oral administration as well as subcutaneous and intraperitoneal injections with pV compounds. Toxicity of pV was assessed by observing the general appearance and behaviour of the rats.

CHAPTER 3: MATERIAL AND METHODS

pV compounds and vanadate:

Sodium orthovanadate was acquired from Sigma (St. Louis, Mo, USA). Vanadium oxide (V_2O_5) and the ancillary ligands used in the synthesis of the peroxovanadates (pVs) were purchased from Aldrich. The pV compounds were synthesized by Dr. Jesse Ng of the Department of Chemistry, McGill University.

The pV complexes were prepared by dissolving V_2O_5 in aqueous KOH, followed by the addition of 30% H_2O_2 and the ancillary ligand.

The pV compounds employed were:

bpV(phen) = $K[VO(O_2)_2phen] \cdot 3H_2O$;
bpV(pic) = $K_2[VO(O_2)_2(pic)] \cdot H_2O$;
mpV(2,6-pdc) = $NH_4[VO(O_2)2,6\text{-}pdc)H_2O] \cdot H_2O$

bp stands for bisperoxo
mp stands for monoperoxo

All the complex salts readily dissolved in H_2O. In the solid state the complexes, stored at 4^0C in a light-shielded container, appear to be stable indefinitely. Thus, preparations of bpV(phen) >3 years old retained full potency. The compounds were dissoved in phosphate buffered saline (PBS) and the pH was adjusted to 7.4 before administration (Posner et al. 1994).

Insulin:

Beef-pork Ultralente insulin, a long-acting agent, was purchased from Connaught Novo Nordisk Laboratories Ltd., Willowdale, Ontario, Canada.

Animals

BB rats:

BB rats were obtained from Dr. Pierre Thibert, Animal Resources Division, Health and Welfare Canada, Ottawa, ON.

The BB rat was discovered in 1974 in a Wistar-derived commercial breeding colony in Ottawa, Ontario, Canada. Diabetes in BB rats closely resembles human insulin-dependent diabetes mellitus (IDDM). The BB rat is characterized by hyperglycaemia, weight loss, polyuria, severe ketosis, and death unless insulin therapy is initiated. The usual onset of diabetes is around 60-100 days of age. The incidence is 40-70%. Like human IDDM, diabetes in the BB rat is accompanied by autoantibodies to the β cell and by other immune system disorders (55). Diabetic BB rats were treated with subcutaneous injections of beef/pork ultralente insulin whose dosages were adjusted to control glycosuria.

Streptozotocin-diabetic Wistar rats: (STZ-diabetic rats):

Female Wistar rats were purchased from Charles River

Canada Inc. After 3 days of adaptation, diabetes was induced by a tail vein injection of a single dose of streptozotocin (55mg/kg) dissolved in NaCitrate buffer (pH=4.53). Control rats received a PO_4 buffer injection only. The rats were maintained untreated for one week. Glucosuria was checked daily to confirm diabetes, and then blood glucose levels were checked twice after an 8h fast, 3 days apart.

Both BB and STZ-diabetic rats were housed in metabolic cages up to a maximum of 4 rats per cage according to the requirements of the experiment. Rats were maintained in a constant temperature (20^{0}C) and humidity (70%) room, in laminar flow hoods, with a fixed cycle of 12h light: 12h darkness (light on 7:00-19:00). Rat Chow (Ralston Purina, St. Louis, Mo, USA) and tap water were provided ad libitum, except when indicated otherwise.

Effects of oral pV compounds in STZ-diabetic rats:

After a 16 hour fast blood glucose was measured using a Companion 2 glucose metre. The rats were divided into several groups matched for body weight and glucose levels. The rats then received one of the following pV compounds in the drinking water in concentrations described in the appropriate figure legends: bpV(phen), mpV(2,6-pdc), bpV(pic) and Vanadate. Some rats received the medication

with aspartame. The water bottles were protected from light by aluminium foil. The pV solutions were prepared every 3 days. Every day, the amount of water consumed by each rat was measured and recorded. The remaining water was discarded. The bottle was refilled using the refrigerated stock solution. All preparations contained NaCl 80mmol/L for reducing toxic effects of bpV(phen).

Food was provided ad libitum to treated and control rats. Pair-fed rats were added as additional control rats to differentiate the direct effects of treatment from those related to a decreased food intake. In these studies rats were placed in metabolic cages, only one rat per cage. The amount of food eaten by each rat can be measured because of the design of the cage that prevents animals from spilling the powdered food provided. The same amount of food eaten by each rat from the treatment groups was provided to the pair-fed control rats. Pair-fed rats ate all the food provided to them.

Plasma glucose was measured in all rats after an 8 hour fast (fast 8h00-16h00, sample 16h00) twice a week. Glucosuria and Ketonuria were assessed qualitatively at the same times. Body weight, food and water intake were recorded every day.

Effects of Subcutaneous Injections of bpV(phen) in STZ-Diabetic Rats:

The rats were divided into 2 groups matched for body weight and glucose levels. Food was ad libitum. The rats were treated by subcutaneous injection of bpV(phen) 1.5μmol/100g BW/day (n=4, duration = 8 days); bpV(phen) 2.5μmol/100g BW/day (n= 4, duration = 8 days); the injection site was changed each time. The skin condition at the injection sites was observed and recorded daily. Fasting blood glucose and body weight were also recorded daily.

Effects of Intraperitoneal Injections of bpV(phen) in Insulin-deprived BB rats:

To ensure the insulin-deprived state in BB rats, insulin treatment was stopped 16 hour preceding the experiment, at which time their plasma glucose levels exceeded 16.8mmol/L. Treatment was initiated by intraperitoneal injections of bpV(phen) 0.5μmol/100g BW BID (n=4) or phosphate buffer 0.1ml/100g BID, (n=4) alone. Nonfasting blood glucose levels were measured daily (at 16 hour). Glucosuria, ketonuria and body weight were recorded daily. Food was ad libitum.

Effects of bpV(phen) on the Prevention of Diabetes in BB rats:

120 diabetes-prone BB rats (30 days of age) were obtained from Health and Welfare Canada. Fasting blood glucose was measured one and two days before the experiment. The glucose levels were between 4.8 and 7.7 mmol/L. Rats were divided into 4 groups matched for body weight and glucose levels. Treatment was initiated by intraperitoneal injection of bpV(phen) at a dosage of 0.1μmol/100g BW BID (n=30; duration = 130 days); or 0.25μmol/100g BW BID (n=30; duration = 130 days); phosphate buffer 0.1ml/100g BW BID alone (n=30; duration = 130 days)or insulin 7.5 units /kg BW BID (by subcutaneous injection)(n=30; duration = 130 days). When the rats became diabetic, the experimental treatment was continued but insulin treatment was initiated (or adjusted for the insulin group). Food was ad libitum. Fasting tail vein blood was taken without anaesthesia for measurement of insulin, rat C-peptide and plasma glucose levels before treatment and 2 hours after treatment once a month. Nonfasting blood glucose was measured using the Companion 2 glucose metre once a week (before treatment and 2 hours after treatment). Glucosuria and ketonuria were recorded by chemstrip (uG/K chemstrip) once a week (once a day for the rats receiving insulin treatment). Body weight was recorded daily.

Statistical analysis:

Results were given as the mean ± SEM for the indicated

number of rats. Comparisons among pV compounds, vanadate, and control rats were carried out by one-way ANOVA, followed by the Duncan's multiple range test for multiple comparisons, by Chi-square and by T test. Differences were considered statistically significant at $p<0.05$.

CHAPTER 4: RESULTS

Effects of oral pV compounds in STZ-diabetic rats

Effects on glucose levels:

STZ-diabetic rats exhibited stable hyperglycaemia. After treatment with the lower dose of bpV(phen)(0.25mg/ml or less in drinking water), the plasma glucose levels did not show a significant decrease compared with pair-fed control rats. The bpV(phen) treated diabetic rats responded by lowering their blood glucose levels only when the concentration of bpV(phen) increased to 0.75mg/ml. The plasma glucose levels of rats dropped within 3 days after increasing bpV(phen) to 0.75mg/ml, and thereafter, remained stable as long as the treatment was maintained, the plasma glucose levels of pair-fed rats shown decline later than treated rats and then increased close to control levels (Fig.1A). After increasing the dose of bpV(phen) from 0.25 to 0.75mg/ml the average plasma glucose level decreased from 20.1 to 9.5mmol/L ($p<0.05$) in treated rats, with only a slight decrease observed in pair-fed rats (from 18.6mmol/L to 16.0mmol/L)(Table 2a). Initial doses of mpV(2,6pdc), and bpV(pic) had little effect on plasma glucose. When increased dosages of these two compounds were given, plasma glucose started to decline in both treated and pair-fed rats (Table 2a,b,c).

The blood glucose levels in vanadate 0.25mg/ml treated and pair-fed control rats was significantly lower than in STZ-diabetic control rats ($p<0.05$). However no significant difference was observed between vanadate treated and pair-fed rats. This indicated that the lower glycemic effects of vanadate was attributable to reduced food intake (Table 1).

Effects on food intake:

The induction of the diabetic state resulted in increased food intake (hyperphagia)(Fig.1B). The food consumption in STZ-diabetic control rats was higher than in the non-diabetic controls($p<0.05$) (Fig.1B). Administration of pV compounds in diabetic rats inhibited food intake, compared to untreated-control STZ-diabetic rats ($p<0.05$)(Fig.1B, table 2a,b,c). Following the increase in concentration of pV compounds, a more severe decrease in food intake was observed (Table 2a,b,c). mpV(2,6 pdc) produced the strongest inhibition of food intake when compared in both bpV(phen) and bpV(pic) treated groups ($p<0.05$). After the same concentration of pV compounds treatment (0.25mg/ml in drinking water), the average food intake (g/rat/day) was 28.4g in bpV(phen) group, 26.5g in bpV(pic) group and 21.4g in mpV(2,6 pdc) group ($p<0.05$)(Table 2a,b,c). Vanadate appeared to cause a stronger inhibition of food intake than bpV(phen)(Table 1).

Effects on water intake:

STZ-diabetic rats receiving pV compounds dissolved in their drinking water rapidly (within the first day) and markedly reduced their water intake ($p<0.05$)(Fig.1C). Increasing the concentration of pV compounds caused an even more severe decrease in water intake(Fig.1C). The average water intake was significantly more inhibited in rats treated with pV compounds than in control rats ($p<0.05$). As with food intake, different pV compounds produced different inhibition of water intake. bpV(phen) inhibited water intake least, while mpV(2,6-pdc) was the strongest ($p<0.05$)(table 2a,b,c). Vanadate was a stronger inhibitor of water intake than bpV(phen). At a concentration of treatment of 0.25mg/ml in drinking water the average daily water intake was 107ml in bpV(phen) treated group, but only 78ml in vanadate treated group (Table.1).

The effects of Aspartame on fluid intake is shown in table 3. Aspartame increased water intake in STZ-diabetic control rats ($p<0.05$). It had no significant increase on water intake of rats treated with bpV(phen) or vanadate, although the tendency was in the same direction.

Effects on body weight:

At the beginning of the study, the diabetic rats were

matched for body weight. Following treatment with bpV(phen) the body weight showed a tendency to be higher than that of pair-fed rats and diabetic control rats (Fig.1D). After treatment with bpV(phen)(0.05 and 0.5mg/ml), the average body weight of rats was significantly higher than that of pair-fed and STZ-diabetic control rats ($p<0.05$)(table, 2a). However, administration of bpV(phen)0.25 or 0.75mg/ml, mpV(2,6pdc), and bpV(pic) had no significant effect on body weight compared to STZ-diabetic rats although a tendency to increase body weight was observed (Table 2a). The average body weight was not different between the vanadate treated rats and their diabetic controls (Table 1).

Effects of subcutaneous injections of bpV(phen) in STZ-diabetes rats:

24 hours after a single subcutaneous dose of bpV(phen) a skin inflammation and ulcers at the site of injection were observed in both bpV(phen)1.5μmol and bpV(phen)2.5μmol treated groups. The subcutaneous administration of bpV(phen)1.5μmol and 2.5μmol did not acutely decrease plasma glucose. This route of administration was therefore abandoned

Effects of intraperitoneal injections of bpV(phen) in

insulin-deprived BB rats:

bpV(phen)(0.5 μmol /100g BW IP BID) allowed insulin-deprived diabetic BB rats to survive in apparent good health for 25 days, but did not decrease plasma glucose. On day 26, the dose of bpV(phen) was increased to 0.75μmol /100g BW IP BID in an attempt to reduce glucose levels. This led to skin ulcerations at the site of injection, inhibition of food and water intake, a deterioration in the general state of the rats and death. The higher dose of bpV(phen) still did not decrease plasma glucose($p>0.05$). In the absence of insulin ketone levels in bpV(phen) treated rats was increased only slightly. Following treatment with bpV(phen) for another three days the ketosis was corrected and the ketone levels remained normal until the end of the experiment, though no significant change in plasma glucose levels was observed. Insulin withdrawal resulted in hyperglycemia and increased ketone levels by the next day in the diabetic rats. Ketone levels went up very quickly and much higher than that of bpV(phen)treated rats ($p<0.05$). Without insulin treatment the control rats became very sick and started to die after 4 days. All control rats died within 17 days (Fig.2A,B).

When the experiment was repeated, the ketone levels were decreased again following bpV(phen) treatment. Glucose

levels showed a tendency to decline, but were not significant. However severe toxic effects occurred, including decreased food and water intake, diarrhea, bloody eyes and nose, dehydration, poor general state and death. The death rate was higher in bpV(phen) treated rats than that in control rats. Without insulin treatment control rats became very sick soon with higher glucose and ketone levels. The rats started to die after 2 days, three of them died within one week (Fig.3A,B).

Attempt to Prevent diabetes in Diabetes-Prone BB rats by bpV(phen) treatment:

Effects on the onset of diabetes:

bpV(phen) accelerated rather than delayed the onset of diabetes in diabetes-prone BB rats. Insulin therapy delayed the onset, but did not reduce the final incidence of diabetes. The rats started to become diabetic from age 55 days in bpV(phen) 0.25μmol treated BB rats; from age 57 days in bpV(phen) 0.1μmol treated BB rats; from age 65 days in insulin treated BB rats; from age 51 days in phosphate-buffer treated BB rats. The final incidence of diabetes was 78% in bpV(phen) 0.25μmol/100g BW IP BID treated BB rats; 62 % in bpV(phen) 0.1μmol/100g BW IP BID treated BB rats; 54 % in insulin treated BB rats and 52 % in phosphate-buffer treated BB rats (Fig.4).(Comparisons

among bpV(phen) and control rats were carried out by Chi-square. $p>0.05$ because too many rats were dead in treated.)

Effects on insulin and C peptide levels:

bpV(phen) treatment resulted in a lowering of insulin levels and inhibition in endogenous insulin secretion. The levels of insulin and C peptide were significantly lower in rats treated with bpV(phen) 0.25 μmol/100g BW IP BID than in control rats ($p<0.05$). C peptide levels were significantly higher in rats treated with bpV(phen) 0.1 μmol/100g BW IP BID. Insulin therapy resulted in markedly elevated insulin levels and in an almost complete suppression of C-peptide levels 2 hours post injection ($p<0.05$)(Fig.5A,B).

Effects on insulin requirement:

Once the diabetes-prone BB rats were diabetic, the insulin dose required to maintain the aglycosuric state of the rats was lower in both bpV(phen) treated groups compared to either the insulin group or the PO_4 control group ($p<0.05$)(Fig.5C).

Effects on plasma glucose levels:

Intraperitoneal injection of bpV(phen) treatment did not

cause a significant decrease in plasma glucose in BB rats. Insulin caused a marked reduction in plasma glucose levels post injection ($p<0.05$)(Fig.6).

Effects on body weight:

bpV(phen) did not have a significant influence on body weight gain with age compared with diabetic control in BB rats. Insulin administration resulted in a greater weight gain with age (Fig.7)($p<0.05$).

Effect on mortality:

Following intraperitoneal injection of bpV(phen) treatment, the death rate was increased, especially in the higher dose group. In most cases, death was preceded by an enlarged abdomen in bpV(phen) treated rats. The final death rate was 83% in bpV(phen)(0.25μmol /100 BW BID) treated group; 50% in bpV(phen)(0.1 μmol /100 BW BID) treated group; 40% in insulin treated control group and 30% in PO4 treated control group. When we increased the bpV(phen) dosage from 0.25 to 0.35 μmol/100 BW BID twice(during experiment days 21 to 27 and 39 to 46) the death rate increased markedly(Fig.8).(Comparisons among bpV(phen) and control rats were carried out by Chi-square. $p>0.05$ because too many rats were dead in treated.)

CHAPTER 5: DISCUSSION

Since 1987, studies have suggested that pV compounds are more powerful and less toxic antidiabetic agents than vanadium both in vitro (32,51,53) and in acute in vivo studies (17,41). In this thesis work, I was interested in determining whether pV compounds had potent metabolic effects and lower toxicity than vanadate under long-term treatment. The oral route, more appropriate for clinical use, has been used in almost all long-term in vivo vanadium studies. Previous studies also shown that the oral administration of vanadate was safer than the intraperitoneal or intravenous routes (55). Therefore, we first used the oral administration route in our longer-term studies.

Previous studies demonstrated that vanadate in the drinking water of STZ-diabetic rats normalized blood glucose levels, reduced the symptoms such as hyperphagia, polyuria, and improved the depressed renal and cardiac functions associated with diabetes (29,56). The basic mechanism of the antidiabetic action of vanadate in vivo is generally attributed to its insulin mimetic actions. However, the inhibition of feeding (45,46,47) and of the N_a^+- glucose transporter in the rats' small intestine (57) could also play a role in lowering blood glucose levels.

In order to circumvent these factors, we included in our studies pair-fed control rats to separate the direct effects of pV compounds from their effects on food intake.

The plasma glucose levels of rats dropped within 3 days after increasing bpV(phen) to 0.75mg/ml from 0.25mg/ml, and thereafter, remained stable as long as the treatment was maintained (Fig.1A). The average blood glucose level of STZ-diabetic rats dropped 53% following treatment with bpV(phen) 0.75 mg/ml in drinking water. Untreated pair-fed STZ-diabetic rats restricted to the same amount of food intake as the bpV(phen) treated STZ-diabetic rats did not show identical lowering of blood glucose levels (Table 2a, Fig. 1A). Therefore, this glucose fall in the bpV(phen) 0.75 mg/ml treated group can be attributed almost entirely to the insulin mimetic effects of bpV(phen). mpV(2,6-pdc), bpV(pic) administered in drinking water had no direct effects on plasma glucose levels (table 2b,c).

As shown in table 1, the plasma glucose levels in both vanadate (0.25 mg/ml) treated and its pair-fed rats were significantly lower than in the STZ-diabetic control rats, but not significantly different between vanadate treated and pair-fed rats. This indicated that the glycemic lowering effects of vanadate was caused by reduced food intake.

Though vanadium administered orally was safer than when given by other routes, the doses of vanadium used orally are still higher than those considered to be toxic in normal rats (55). In recent years the toxic side effects noted with chronic oral administration of vanadate in animals argues against its use in humans with diabetes. It was reported that the mortality rate was significantly higher, food and fluid intake, and weight gain were lower in vanadium-treated rats than in untreated control rats (48). Vanadate also depressed critical enzymes in the liver of diabetic rats and accumulated in liver, kidney, heart, bone and muscle. Kidney and bone were reported to be the main sites of vanadium accumulation (45,46,47). The intravenous administration of sodium orthovanadate or vanadyl sulfate produced mortality before any significant reduction of blood sugar levels was observed (41).

Compared with vanadate, pV compounds added to the drinking water of diabetic rats caused less toxic effects on inhibiting food and water intake although the same concentration of vanadate and bpV(phen)(0.25mg/ml) were administered to the rats (table 1). All STZ-diabetic rats receiving water containing the pV compounds rapidly and markedly reduced their food and water intake on the first day. Increasing the concentration of pV compounds caused an even more severe decrease in food and water intake

(fig.1B,C). bpV(phen) had the least inhibition on food and water intake among pV compounds (table 2a,b,c). The mechanism leading to an inhibition of food and water intake by pV compounds is not clear. Our observation of the dilated stomachs full of food in some rats that died while being treated with pV compounds led us hypothesize that the pylorus might be stimulated to contract by these compounds since tyrosine phosphorylation caused by vanadate was an important mechanism for stimulation of smooth muscle contraction (49). This contraction of the pylorus might have contributed to food and water retention in the stomach which might subsequently be responsible for the diminished water and food intake. Though the food intake was lower for most of the treated rats the body weight appeared to be higher than that of the diabetic control or pair-fed rats (Table 2a,b,c). The body weight of bpV(phen) 0.5mg/ml treated rats was higher than that of the diabetic controls ($p<0.05$). An anabolic effect of bpV(phen) might have contributed to the increased body weight.

The diabetic rats' spontaneous intake of water, containing various concentrations of pV compounds, was highly variable. When we increased the concentration of bpV(phen) from 0.25 to 0.75 mg/ml, the significant decrease in water intake that occured prevented us from increasing the dose

administered (Fig. 1c). The taste of the pV compound may have contributed to the decreased fluid consumption. The sweet taste of aspartame added to the pV compound preparation did not prevent the decreased water intake caused by bpV(phen) and vanadate (Table 3). The poorly controlled rats drank the most and hence had the highest overall pV intake. A larger volume of pV consumed might have promoted a more pronounced diuresis and thus caused a greater elimination of pV, since it was previously reported that vanadium was rapidly excreted by the kidneys (58). In order to avoid these confounders related to the administration of pV compounds in the drinking water and ensure a standardized daily dose of pV compounds and to prevent the possible degradation of bpV(phen) in the low pH of the stomach we chose to provide the pV compounds by subcutaneous and intraperitoneal injections instead of the oral administered route.

It was reported that subcutaneous injections of bpV(phen) induced a significant ($p < 0.01$) decrease in plasma glucose (41). In our long-term studies, a subcutaneous injection of bpV(phen) 1.5 μmol and bpV(phen) 2.5 μmol had no significant effects on blood glucose, but caused skin inflammation and an ulcer at the site of the injection, which prevented us from using the subcutaneous injection route for long-term studies. The skin ulcers at the site

of the injection were possibly induced by local vasoconstriction since vanadate caused severe vasoconstriction (31).

Assia Shisheva et al.(1994) reported that a single intraperitoneal injection of pervanadate had a dramatic effect in improving glucose homeostasis in STZ diabetic-rats at doses corresponding to only 700 μg vanadium/kg body weight (14). The effect was superior to that achieved by the acute administration of submaximal doses and comparable to moderate doses of crystalline insulin. The intraperitoneal administration of pV compounds to insulin deprived BB rats over a 3-day period produced a persistent lowering of both blood glucose and ketone body levels (17). As expected, in our study bpV(phen) had insulin-mimetic actions in insulin-deprived BB rats when injected intraperitoneally. To ensure the insulin-deprived state of the BB rats, insulin was stopped 16 hours before bpV (phen) was started. Following bpV(phen) treatment by intraperitoneal injection, rats appeared to be in good health for 25 days, and the ketonuria was corrected although plasma glucose did not decrease. As the rats appeared to be still in good health on day 25, we increased the dose to 1.5 μmol/100g BW/day, in the hope of decreasing plasma glucose levels. After increasing the dose, the rats showed a deterioration in their general

state. At the same time, ulcers at the injection sites were observed and still no significant change in plasma glucose levels was observed. These results indicate that a dose of 1.5 μmol/100g BW/day intraperitoneally is above the maximum dose that rats can tolerate (Fig. 2A).

Without insulin treatment the control rats became very sick and started to die after 4 days. All the control rats died within 17 days (Fig. 2A). Insulin withdrawal resulted in an immediate hyperglycemia and increased ketone levels by the next day in the control diabetic rats. The ketone levels went up very quickly and remained higher until ketoacidosis became too severe to be detected (Fig.2B). The limit of detection of the Chemstrip used for acetoacetic acid was 5-10 mg/dl/1 urine, and β-hydroxybutyric was not determined. During the progression of ketoacidosis, accelerated fatty acid oxidation in liver mitochondria generates excessive quantities of acetoacetate and ion H^+. Acetoacetate can be reduced to β-hydroxybutyric acid and this rise in the β-hydroxybutyric acid to acetoacetate acid ratio results in undetectable levels of acetoacetic acid.

The study described represents what we believe to be so far the longest treatment of insulin-deprived BB rats with an agent other than insulin. It is consistent with the

view that bpV(phen) exerts its insulin mimetic action by acting on the insulin receptor and post receptor pathway of the peripheral target tissue. However, this treatment did not appear to improve the plasma glucose levels.

When we repeated this experiment, bpV(phen) again corrected ketone levels in insulin-deprived diabetic BB rats (Fig.3A,B) , but caused severe toxic effects which included decreased food and water intake, diarrhea, bloody eyes and nose, dehydration, poor general state and death. Intraperitoneal injections of bpV(phen) 0.5μmol/100g BW BID is also above the maximum dose that rats can tolerate (Fig. 3A).

Unlike STZ-rats, BB diabetic rats are absolutely dependent on insulin for survival, their diabetes being caused by a permanent autoimmune destruction of the β-cells. Vanadate also has insulin-like effects on BB diabetic rats, but it can only potentiate insulin, not replace it entirely. S.Ramanadham, et al. 1990 indicated that the administration of vanadyl (0.25 mg/ml) in the drinking water of the animals resulted in a gradual decrease in the dosage of insulin required to maintain the rats nonglycosuric, which neither this minimal dose of insulin nor vanadyl(0.25 mg/ml) could produce alone (59). The same result was found by Maryl et al.1992. The insulin dose

required to maintain the aglycosuric state of the rats decreased from an initial mean of 12.4 units/kg to a nadir of 3.4 units/kg during the 6 months of vanadate treatment in drinking water. However, the need for insulin remained, both with respect to the control of glycemia and the anabolic effect of insulin (60).

Though the dose (0.1 or 0.25 μmol/100g BW IP BID) was not sufficient to decrease the glucose levels significantly in BB rats (Fig.6), insulin-mimetic effects particularly the inhibition of insulin secretion by β-cells could be seen in BB rats. After bpV(phen)(0.25μmol/100g BW IP BID) treatment, the insulin and C peptide levels were significantly reduced (Fig.5A,B). When the rats became diabetic, we started the insulin treatment while continuing the bpV(phen) treatment. The insulin dose required to maintain the aglycosuric state of the rats decreased significantly in the two bpV(phen) treated groups, especially in the higher dose group (fig.5C). These results indicate that bpV(phen) not only inhibits endogenous insulin secretion, but also increases the potency of exogenous insulin. In bpV (phen)(0.1μmol/100g BW IP BID) treated rats the insulin levels and insulin requirements were reduced significantly($p<0.05$) but the C peptide levels were increased (Fig.5B). The high C peptide levels were caused by a temporary rise of C peptide levels

in some rats early in the experiment. After 2 weeks of bpV(phen)(0.1μmol/100g BW IP BID) treatment, the C peptide levels of some rats were higher and then decreased to control levels, and remained stable until the end of experiment. We do not have a good explanation for this temporary rise of the C peptide following bpV(phen) (0.1μmol/100g BW IP BID) for two weeks.

Vanadate caused an increase in insulin level in STZ-diabetic rats (55). Brichard (1988) had previously reported significantly greater pancreatic insulin levels in STZ-diabetic rats after 60 days of vanadate treatment (61). Ramanadham (1989) reported that vanadate decreased the serum glucose levels, and at the same time enhancing insulin levels up to 133% in STZ-diabetic rats (62). High insulin levels were also found in many other studies (55). The higher insulin level suggested that the STZ-diabetic rats, unlike the BB rats did have some residual insulin which must have been secreted from regenerated β-cells.

Type I diabetes could be prevented by inhibiting endogenous insulin secretion (15,54). A possible mechanism was through protection from immune injury by inhibiting β cell secretion, and probably antigen exposure. Since pVs had powerful insulin-mimetic effects and could inhibit endogenous insulin secretion (17), we assumed that bpV

(phen) might be able to reduce the incidence of diabetes.

bpV(phen) administered intraperitoneally was ineffective in preventing diabetes and accelerated rather than delayed the onset of diabetes in BB rats (Fig.4). Activated T cells play a crucial role in triggering diabetes (55). bpV(phen) might stimulate T cell activity by inhibiting its PTPases since PTPases were negative regulators of T cell activation and pV compounds were powerful PTPase inhibitors in T cell (63). This may explain the higher incidence of diabetes caused by bpV(phen). Therefore, pV compounds should be used for the treatment of diabetes instead of prevention of diabetes unless new pV compounds are developed which can specifically act on insulin receptor PTPase. Insulin delayed the onset of diabetes, but did not decrease its incidence (Fig.4).

bpV(phen) did not have any significant influence on body weight gain with age when compared with control BB rats. Insulin administration resulted in greater weight gain with age (Fig.7).

Intraperitoneal injection of bpV(phen) caused severe toxic side effects while producing the insulin mimetic effects in BB rats. Following intraperitoneal injection of bpV (phen) the death rate was increased, especially in the

higher dose group (Fig.1, Fig.8). In most cases, death was preceded by an enlarged abdomen in bpV(phen) treated rats. Our observation of dilated stomachs and intestine full of food and water in some rats that died while being treated with pV compounds led us to hypothesize that the pyloric sphincters might be stimulated to contract by these compounds, since tyrosine phosphorylation caused by vanadate was an important mechanism for stimulation of smooth muscle contraction (49). This hypothetical contraction of the pylorus caused by bpV(phen) might contribute to food and water retention in the gastrointestinal tract. The precise cause of the death of the rats died was not identified. The major cause of death in most insulin treated rats was hypoglycaemia.

CHAPTER 6: SUMMARY AND CONCLUSION

In summary, the present studies provide evidences for in vivo action or potentiation of the effects of insulin in diabetic animals by vanadate and pV compounds under both chronic and acute condition. Furthermore, we demonstrated that long-term oral bpV(phen) treatment resulted in a lowering blood glucose level in STZ-diabetic rats, with less toxic effects than vanadate. bpV(phen) was the first agent other than insulin that was able to maintain the insulin-deprived diabetic BB rats in apparently good health without ketonuria for 25 days by intraperitoneal injection (IP). Although IP injection of bpV(phen) was ineffective in reducing glucose levels in diabetic BB rats it caused significant decreases of insulin levels, C peptide levels and in insulin dose required to maintain the aglycosuric state of these rats. These observations are consistent with a mechanism of bpV(phen) action at the tissue levels. The exploration of the properties of pV compounds may give rise to insulin substitutes which could be orally administered for the treatment of diabetes and may also help elucidate both the mechanisms of insulin action and the cause of diabetes in the future. However Intraperitoneal injections of bpV(phen) also caused toxic side effects. The toxic effects of bpV(phen) included inhibition of food and water intake, increased mortality,

and acceleration of onset of diabetes presumably by activating T cells. Further studies are required to identify second generation pV compounds with better therapeutic/toxicity ratios and to find ways of delivering pVs orally. This would permit more extensive long term studies on the efficacy of these agents in the treatment of the insulinopenic diabetic state.

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Table. 1: Effects of Oral (in drinking water) bpV(phen) and Vanadate In STZ-Diabetic Rats

GROUP	GLUCOSE (mmol/l)	FOOD INTAKE (g)	WATER INTAKE (ml)	BODY WEIGHT (g)	DOSE INTAKE (mg)
bpV(phen) 0.25 mg/ml	15.5±0.91 (b)	21.8±0.51 (c)	107.4±4.24 (a,b,c)	184.1±4.84 (a)	26.86±1.06 (66.44 µmol)
Pair-fed	13.64±1.11 (c)	20.0±0.56 (c)	135.5±8.33 (c)	161.9±5.24	
bpV(phen) 0.1 mg/ml	17.73±0.63 (b)	27.5±1.36 (b)	187.4±6.98 (a,b,c)	189.5±5.20	9.37±0.35 (23.18 µmol)
Pair-fed	16.09±0.98	24.2±0.91	163.2±8.52 (c)	172.2±5.30	
vanadate 0.25 mg/ml	10.42±1.07 (c)	19.6±0.87 (c)	78.3±5.92 (a,c)	177.3±4.77	19.58±1.48 (106.5 µmol)
Pair-fed	11.56±1.05 (c)	18.3±0.66 (c)	123.8±7.50 (c)	167.1±5.58	
Control STZ-diabetic rats	17.94±0.96	27.1±0.63	208.2±1.80	175.6±5.93	
Control nondiabetic rats	5.31±0.19	17.8±0.99	55.6±2.42	240.3±4.90	

Data shown as mean ±SEM (n=8). Statistical differences between the means of the various groups were evaluated using .ANOVA followed by Duncan's test. a = $p<0.05$ vs pair-fed, b = $p< 0.05$ vs vanadate, c = $p<0.05$ vs STZ-diabetic control rats. The experiment duration : 10 days

Table. 2a: Effects of Oral (in drinking water) bpV(phen) in STZ-Diabetic Rats

Groups	Time Treated (day)	Glucose (mmol/l)	Food Intake (g/day)	Water Intake (ml/day)	Body Weight (g)	Dose Intake (mg/day)	Vanadium Ingested (mg/day)
bpV(phen) 0.05 mg/ml	23	22.1±0.6	35.3±0.9 (a,c)	215.6±9.8	241.2±4.4 (c)	10.2±0.57 (25.23 µmol)	1.29
Pair-fed	23	20.5±1.1	33.2±0.95	216.9±9.1	220.0±9.7		
bpV(phen) 0.25 mg/ml	39	20.1±0.6	28.4±1.7	159.9±8.0 (c)	230.3±13.1	42.3±2.18 (104.63µmol)	5.3
Pair-fed	39	18.6±0.6	28.3±1.6	190.6±7.7 (c)	200.0±13.4		
bpV(phen) 0.5 mg/ml	33	16.7±1.7 (c)	22.2±0.9 (a,c)	94.1±12.1 (c)	227.5±10.9(a,c)	45.3±9.23 (112.10µmol)	5.7
Pair-fed	33	13.0±2.7 (c)	20.0±1.5 (a,c)	117.0±17.0 (c)	180.2±6.3 (a)		
bpV(phen) 0.75 mg/ml	17	9.5±2.0 (a,c)	16.0±1.4 (c)	63.0±4.8 (c)	208.6±10.8	47.4±3.48 (117.24 µmol)	6.0
Pair-fed	17	16.0±0.7 (c)	17.3±1.1 (c)	113.8±11.0 (c)	173.1±12.2		
STZ-diabetic control	57	20.0±0.8	30.1±0.8	233.7±4.1	189.5±8.5		
Non-diabetic control	57	5.3±0.2	21.0±0.4	56.3±7.2	294±9.5		

Data shown as mean ±SEM (n=4). Statistical differences between the means of the various groups were evaluated using ANOVA followed by Duncan's test. a = $p<0.05$ vs pair-fed, c = $p<0.05$ vs control STZ-diabetic rats
The experiment duration : 0-23 days in bpV(phen) 0.05mg/ml treated rats, 24-57 days in bpV(phen) 0.5mg/ml treated rats, 0-39 days in bpV(phen) 0.25mg/ml treated rats, 40-57 days in bpV(phen) 0.75mg/ml treated rats.

Table. 2b: Effects of Oral (in drinking water) mpV(2,6-pdc) In STZ-Diabetic Rats

Groups	Time Treated (day)	Glucose (mmol/l)	Food Intake (g/day)	Water Intake (ml/day)	Body Weight (g)	Dose Intake (mg/day)	Vanadium Ingested (mg/day)
mpV(2,6-pdc) 0.05	23	21.5±0.8	31.5±1.3	207.4±8.9 (a)	201.1±9.4	10.37±0.45 (32.60 µmol)	1.7
Pair-fed	23	19.6±1.0	29.5±1.1	178.2±11.6 (c)	193.4±10.9		
mpV(2,6-pdc) 0.25	57	17.9±1.0 (c)	21.4±1.1 (c)	112.2±7.6 (c)	208.4±10.5	28.05±1.9 (88.18 µmol)	4.5
Pair-fed	57	17.4±2,0 (c)	22.0±0.9 (c)	134.1±11.8 (c)	185.6±5.6		
mpV(2,6-pdc) 0.5	33	13.0±1.7	12.5±2.3 (a,c)	48.4±9.2 (a,c)	173.7±12.9	8.40±3.48 (26.41 µmol)	1.3
Pair-fed	33	12.9±4.7	16.6±1.7 (a,c)	114.4±10.8 (c)	168.5±11.1		
STZ-diabetic control	57	20.0±0.8	30.1±0.8	233.7±4.1	189±8.5		
Non-diabetes control	57	5.3±0.2	21.0±0.4	56.3±7.2	294±9.5		

Data shown as mean ±SEM (n=4). Statistical differences between the means of the various groups were evaluated using ANOVA followed by Duncan's test. a = $p<0.05$ vs pair-fed, c = $p<0.05$ vs control STZ-diabetic rats.
The experiment duration : 0-23 days in mpV(2,6-pdc) 0.05mg/ml treated rats, 24-57 days in mpV(2,6-pdc) 0.5mg/ml treated rats, 0-57 days in mpV(2,6-pdc) 0.25mg/ml treated rats.

Table.2c: Effects of Oral (in drinking water) bpV(pic) In STZ-Diabetic Rats

Groups	Time Treated (day)	Glucose (mmol/l)	Food Intake (g/day)	Water Intake (ml/day)	Body Weight (g)	Dose Intake (mg/day)	Vanadium Ingested (mg/day)
bpV(pic) 0.025	23	20.2±0.5 (a,c)	33.0±1.3	218.2±9.5 (a,c)	219.1±15.2	5.45±0.24 (14.84 µmol)	0.76
Pair-fed	23	21.4±0.6	32.0±0.9	198.2±12.3 (c)	214.3±7.7		
bpV(pic) 0.125	39	21.4±1.4	31.0±2.1	178.8±19.6 (a,c)	231.0±14.5	23.1±2.45 (62.90 µmol)	3.2
Pair-fed	39	20.0±1.2	29.2±1.2	211.3±13.7 (a,c)	206.5±3.9		
bpV(pic) 0.25	33	19.8±1.1	26.5±0.7 (c)	143.6±7.8 (a,c)	227.6±15.1	35.59±0.84 (96.90 µmol)	4.95
Pair-fed	33	20.9±1.3	26.2±0.8 (c)	176.7±7.5 (c)	201.7±10.2		
bpV(pic) 0.375	17	18.8±0.7 (c)	22.3±1.2	118.6±13.3 (a,c)	225.0±16.7	44.9±5.0 (112.25 µmol)	6.24
Pair-fed	17	20.0±1.4	23.1±1.0	167.5±13.2 (c)	193.3±5.5		
STZ-diabetic control	57	20.0±0.8	30.1±0.8	233.7±4.1	189±8.5		
Non-diaebtic control	57	5.3±0.2	20.1±0.4	56.3±7.2	294±9.5		

Data shown as mean ±SEM (n=4). Statistical differences between the means of the various groups were evaluated using ANOVA followed by Duncan's test. a = $p<0.05$ vs pair-fed, c = $p<0.05$ vs control STZ-diabetic rats.
The experiment duration : 0-23 days in bpV(pic) 0.025mg/ml treated rats, 24-57 days in bpV(pic) 0.25mg/ml treated rats, 0-39 days in bpV(pic) 0.125mg/ml treated rats, 40-57 days in bpV(pic) 0.375mg/ml treated rats.

Table. 3: Effects of Aspartame on Water Intake In STZ-Diabetic Rats

Groups	Water Intake (ml/day)	Body Weight (g)
bpV(phen) 0.25 mg/ml	105±30.6 (b, c)	182 ±17
bpV(phen) 0.25 mg/ml +A	142 ±18.5 (b, c)	205± 6.7
Vanadate 0.25 mg/ml	107 ±31.3 (b, c)	182 ±9.3
Vanadate 0.25 mg/ml +A	134 ±6.6 (b, c)	208 ±17.9
STZ-diabetic control	242 ±11 (a)	213 ±12.1
STZ-diabetic control +A	305± 4.0	208± 8.6

Data shown as mean ±SEM (n=4). Statistical differences between the means of the various groups were evaluated using ANOVA followed by Duncan's test. a = $p<0.05$ vs pair-group +A , b = $p< 0.05$ vs STZ-diabetic control , c = $p<0.05$ vs STZ-diabetic control + A .
The experiment duration : 4 days

A = Aspartame

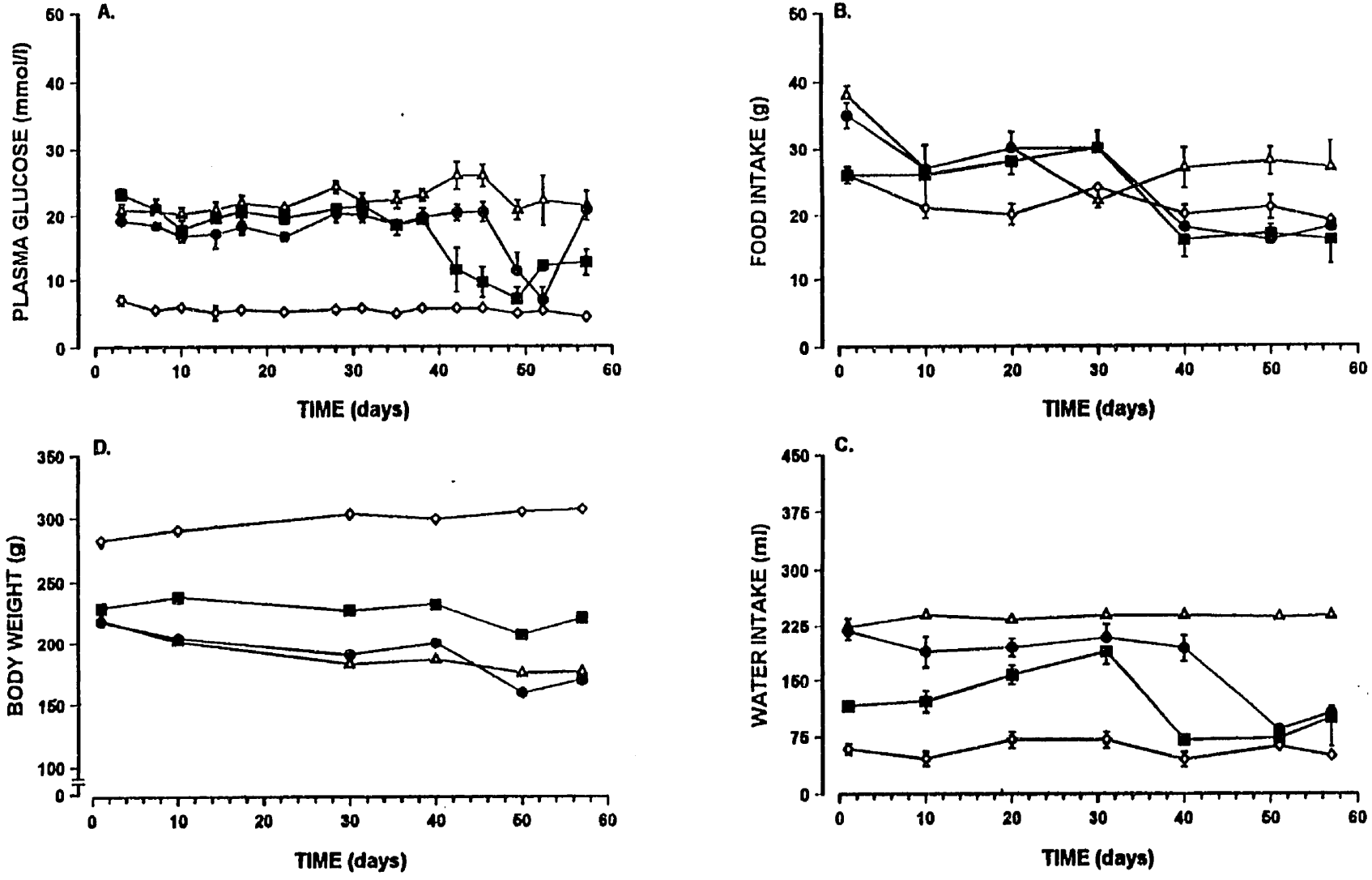


Fig. 1. Effects of oral pV compounds in STZ-diabetic rats. Data shown as mean ± SEM. bpV(phen) 0.25(0-39 days), 0.75 (39-57 days) mg/ml in drinking water (■—■, n=4), Pair-fed to bpV(phen) (●—●, n=4) STZ-diabetic control (▵—▵, n=4), non-diabetic control (◇—◇, n=4).A: Effects of bpV(phen) on plasma glucose levels. B: Effects of bpV(phen) on food intake. C: Effects of bpV(phen) on water intake. D: Effects of bpV(phen) on body weight .

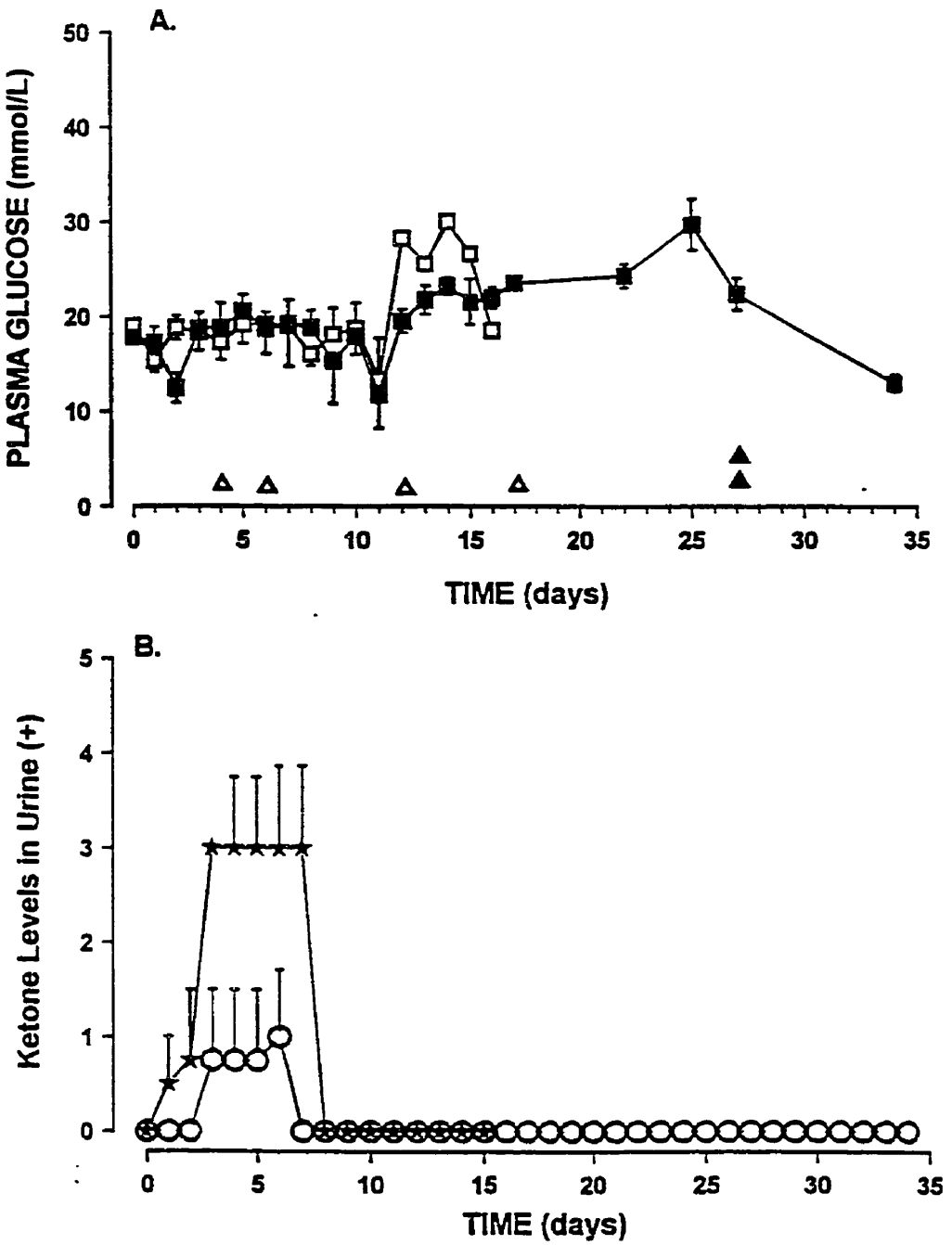


Fig.2. Effects of Intraperitoneal Injections of bpV(phen) in Insulin-deprived BB rats. Data shown as mean ± SEM (n=4). A: Effects of bpV(phen) on Glucose Levels: bpV(phen) 0.5 (day 0-25), 0.75 (day 26-35) µmol/100g BW BID IP (■—■), PBS 0.1ml/100g WB BID IP alone as control (□—□), Dead rats (▲ bpV(phen), Δ control). B: Effects of bpV(phen) on Urine Ketone Levels: bpV(phen) 0.5, 0.75 µmol/100g BW BID IP (○—○), PBS 0.1ml/100g BW BID IP alone as control (★—★).

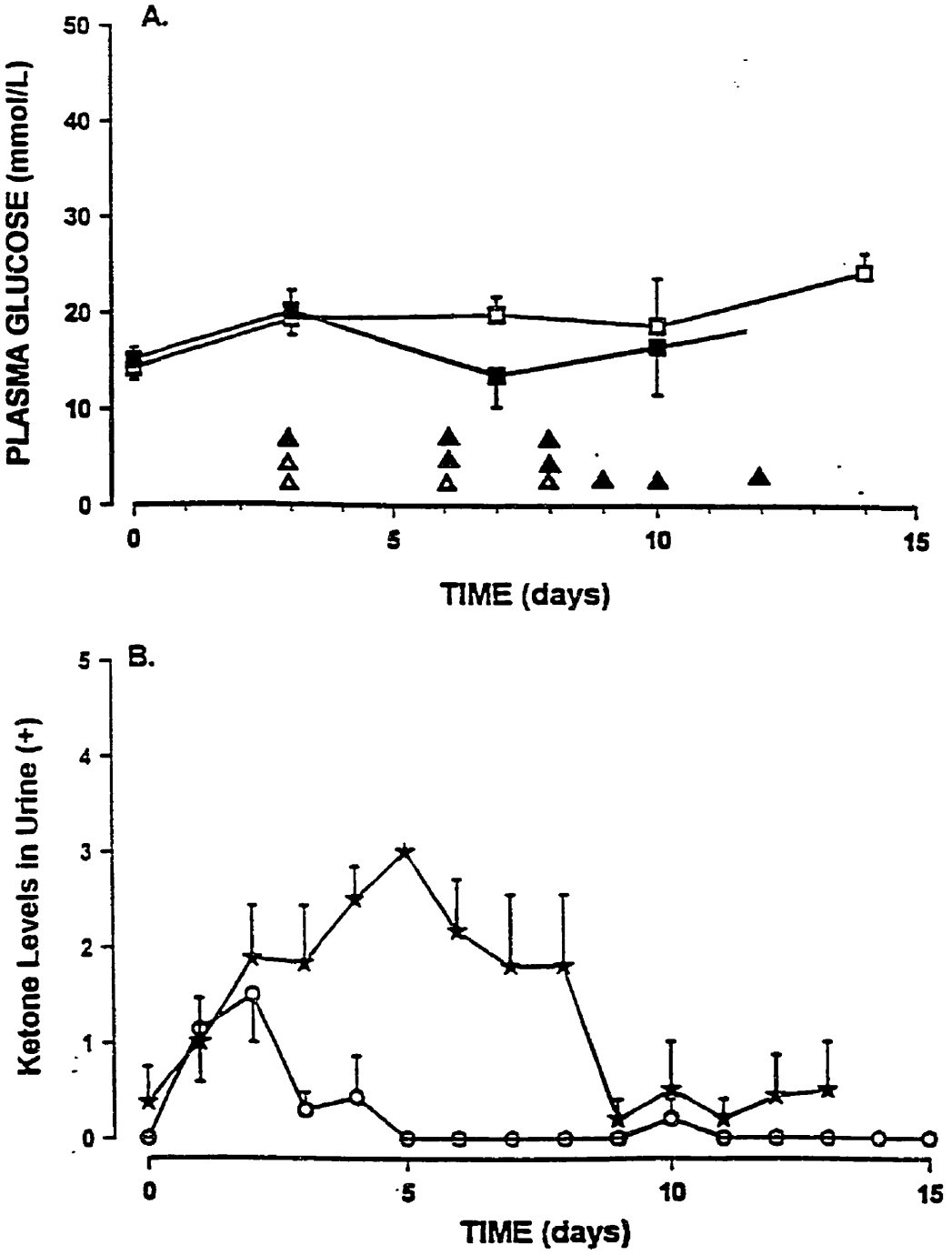


Fig.3. Effects of Intraperitoneal Injections of bpV(phen) in Insulin-deprived BB rats. Data shown as mean ± SEM (n=8). A: Effects of bpV(phen) on Glucose Levels: bpV(phen) 0.5µmol/100g BW BID IP (■—■), PBS 0.1ml/100g BW BID I P alone as control (□—□), Dead rats (▲ bpV(phen), Δ control). B: Effects of bpV(phen) on Urine Ketone Levels: bpV(phen) 0.5µmol/100g BW BID IP (○—○), PBS 0.1ml/100g BW BID IP alone as control (★—★).

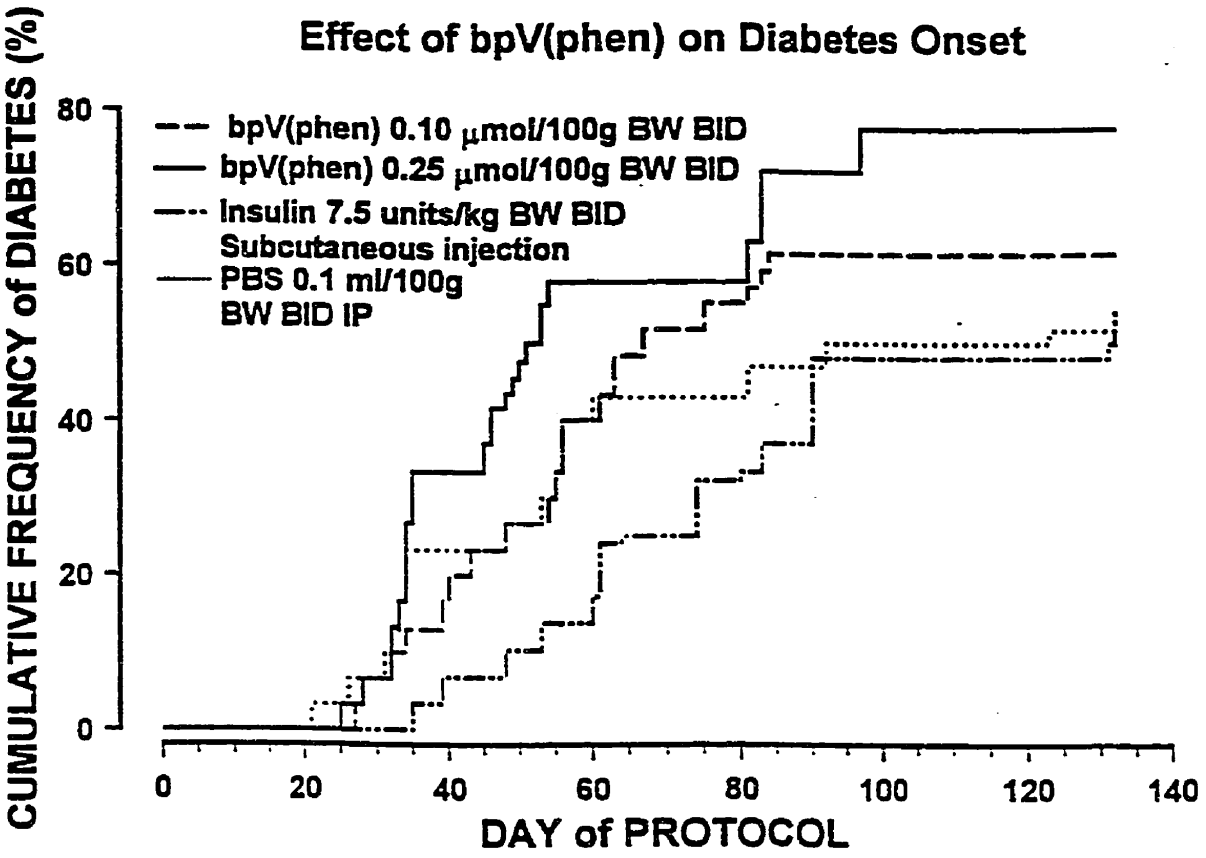


Fig.4.Effects of bpV(phen) on onset of diabetes in Diabetes-Prone BB rats. Data shown as mean ± SEM (n=30).

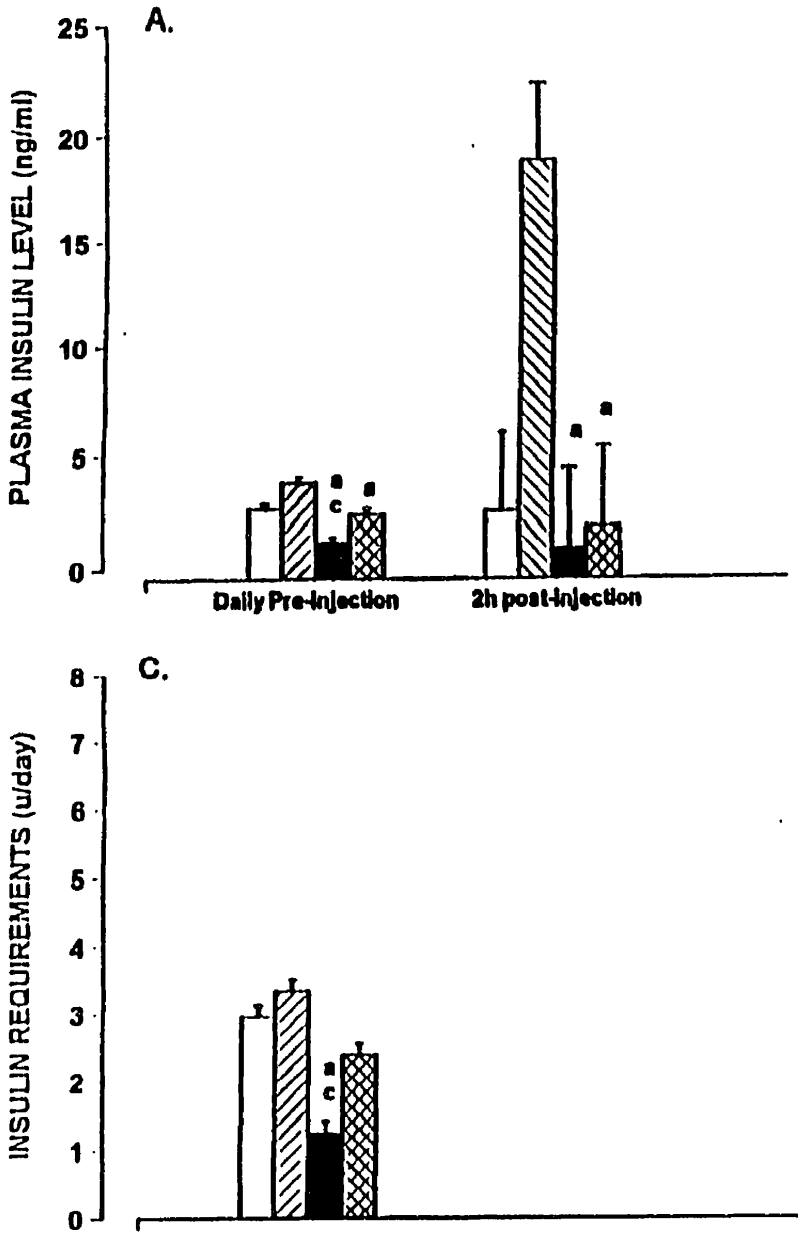


Fig. 5. Effects of bpV(phen) on the Prevention of Diabetes in Diabetes-Prone BB rats. Data shown as mean±SEM. a=p<0.05 vs insulin-control, c=p<0.05 vs po4 control

- ☒ **bpV(phen) 0.1 (μmol/ 100g BW BID IP);**
- ■ **bpV(phen) 0.25 (μmol/100g BW BID IP);**
- ▨ **Insulin 7.5 units/kg BW BID subcutaneous injection;**
- □ **PBS 0.1 ml/100g BW BID IP as control.**

A: Effect of bpV(phen) on plasma insulin levels.
B: Effects of bpV(phen) on C peptide levels.
C: Effects of bpV(phen) on daily insulin requirements.

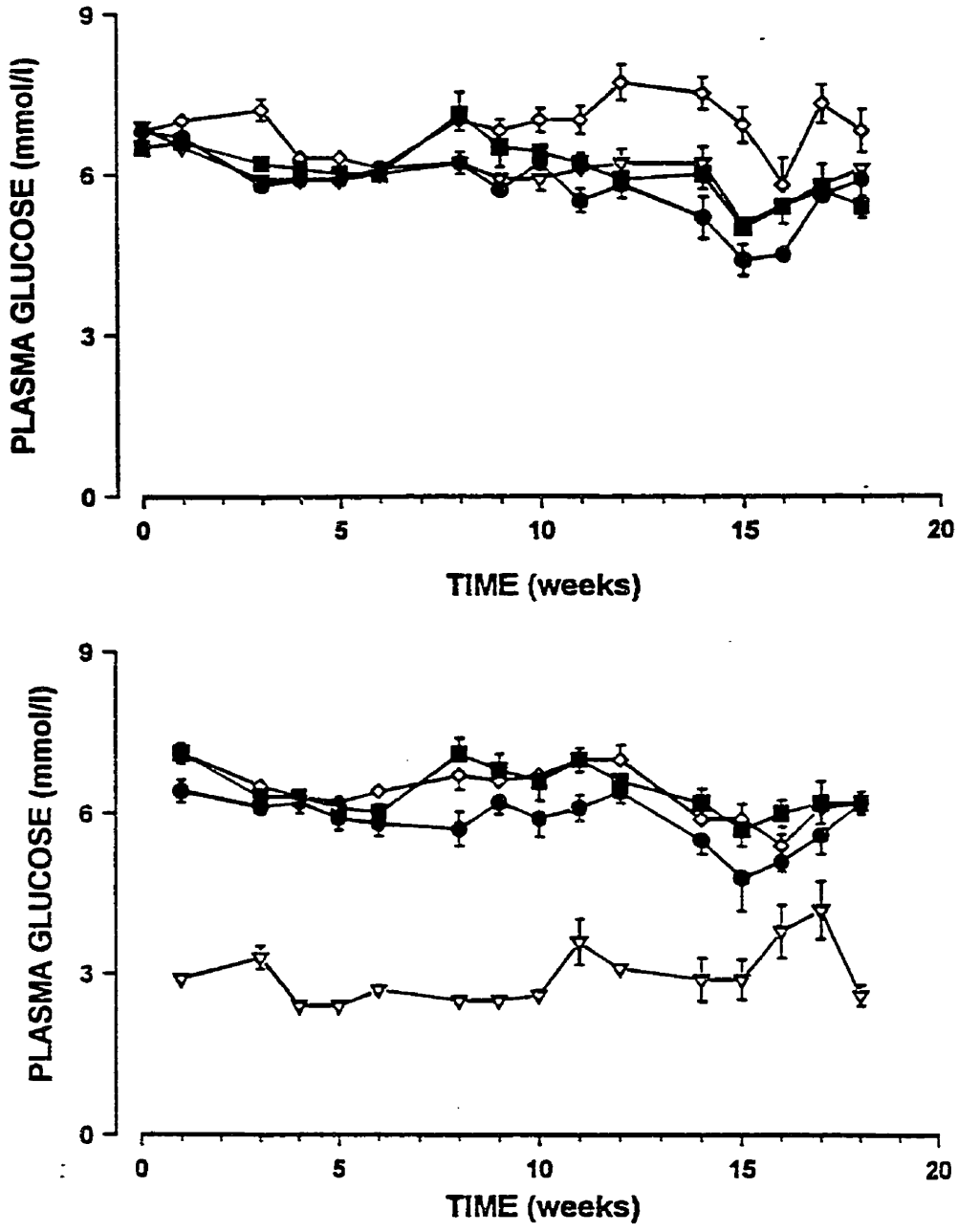


Fig. 6. Effects of bpV(phen) on plasma glucose in Diabetes-Prone BB rats. Data shown as mean ± SEM. bpV(phen) 0.1μmol/100g BW BID IP (■—■, n=30), bpV(phen) 0.25μmol/100g BW BID IP (●—●, n=30), Insulin 7.5 units /kg BW BID subcutaneous injection (▽—▽, n=30), PBS 0.1ml/100g BW BID IP alone as control, (◇—◇, n=30). A: daily preinjection. B: daily 2 hour post injection

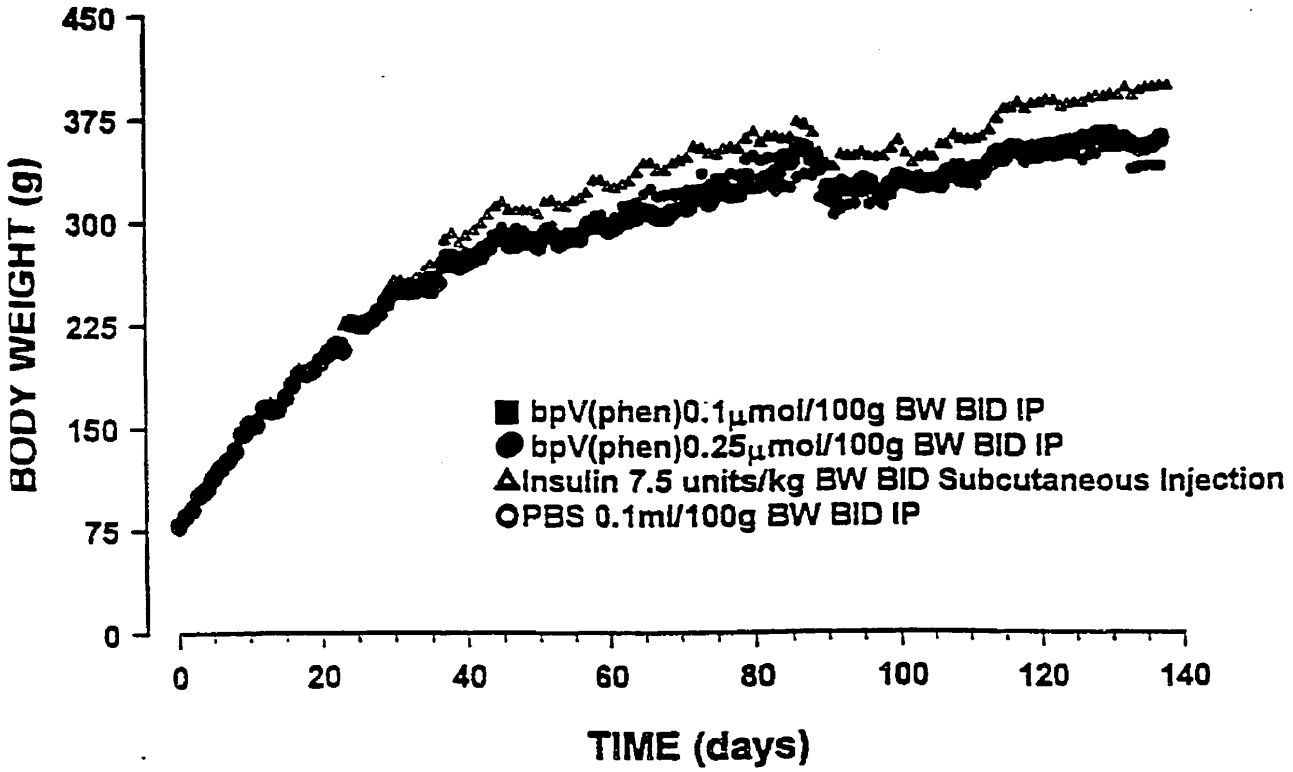


Fig.7.Effects of bpV(phen) on body weight in Diabetes-Prone BB rats. Data shown as mean ± SEM (n=30).

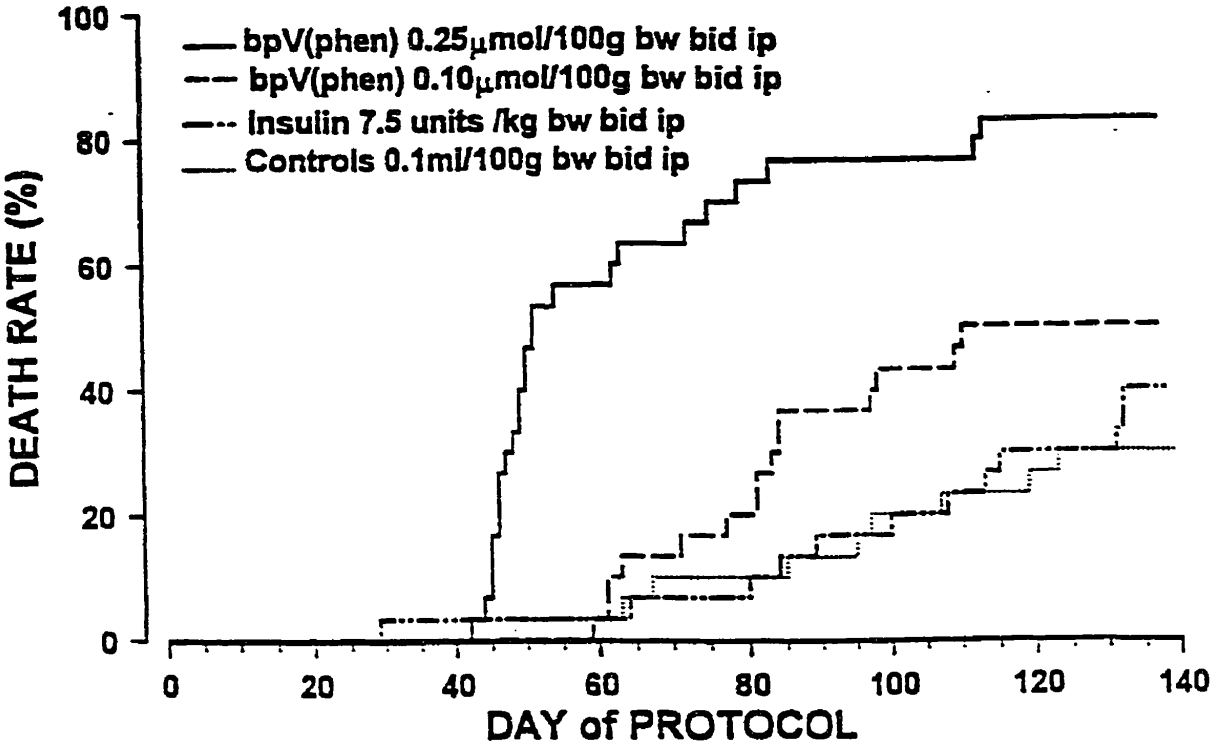
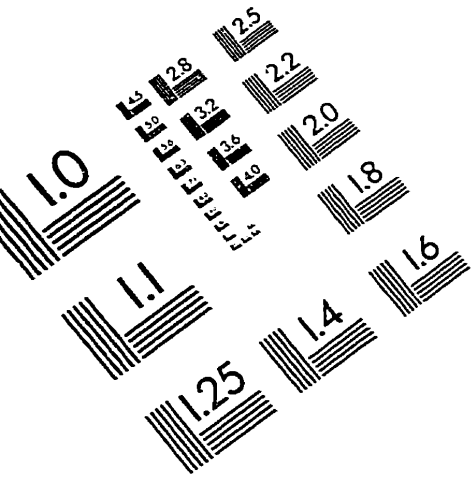
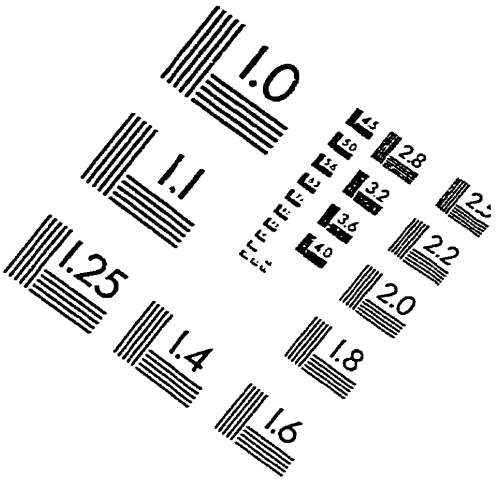
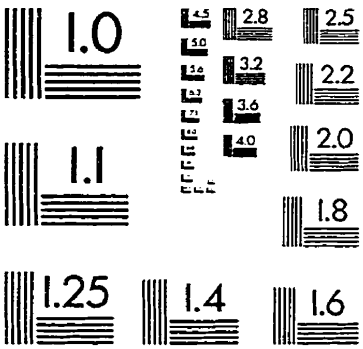


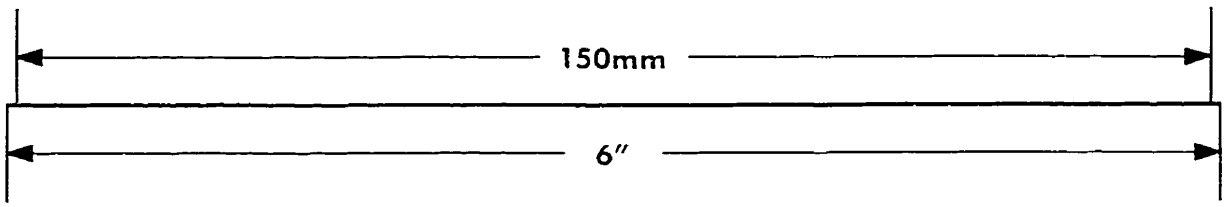
Fig. 8. Effects of bpV(phen) on Mortality in Diabetes-Prone BB Rats. Data shown as mean±SEM (n=30)

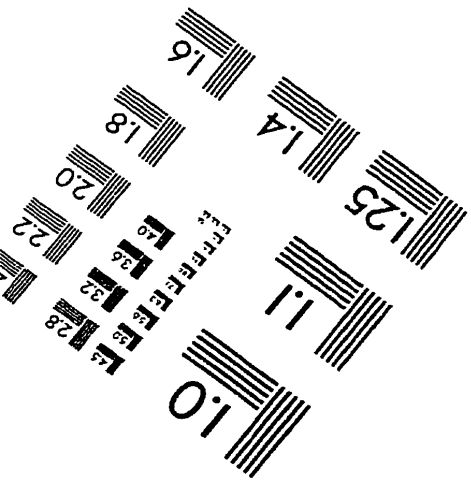
IMAGE EVALUATION
TEST TARGET (QA-3)

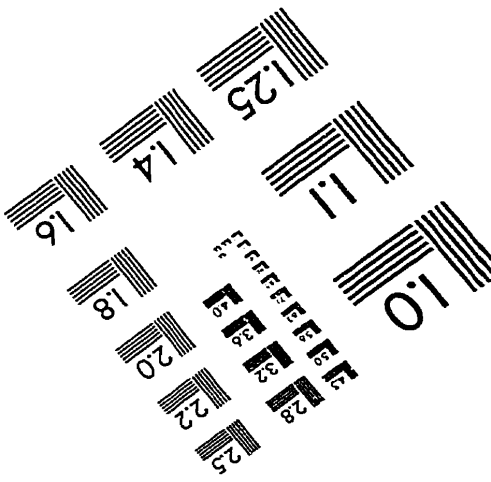












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