ALTERATIONS IN PROTEIN PHOSPHATASE ACTIVITY IN DIABETIC CARDIOMYOPATHY

A Thesis

Presented to the

Faculty of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

by

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

It is now well known that the phosphorylation-dephosphorylation system plays a major role in determining the activities of subcellular organelles in the myocardium. In view of the involvement of protein phosphatase in dephosphorylation, it is possible that changes in subcellular activities in the diabetic heart are associated with alterations in the activity of this enzyme. Since protein phosphatase 1 and protein phosphatase 2A participate in a number of receptor mediated processes for protein dephosphorylation, we examined changes in protein phosphatase 1 and protein phosphatase 2A in diabetic hearts and skeletal muscles. For this purpose, rats were made diabetic by an intravenous injection of streptozotocin (65 mg/kg body wt.) and the hearts were removed 1, 2, 4 and 8 weeks later. At 4 weeks some of the diabetic animals received subcutaneous injection of insulin (3 U/day) for 4 weeks. Protein phosphatase 1 and protein phosphatase 2A activities were measured by using the Upstate Biotechnology kit for protein phosphatase assay. Protein phosphatase 1 in the diabetic heart was increased by 65% and protein phosphatase 2A was increased by 32%. A significant increase in protein phosphatase 1 and protein phosphatase 2A activities was also observed in the skeletal muscles of diabetic animals. These changes in both heart and skeletal muscle were partially reversed upon treating the diabetic animals with insulin. The results indicate an increase in both protein phosphatase 1 and protein phosphatase 2A activities in the diabetic heart. It is suggested that enhanced dephosphorylation of subcellular proteins may play a role in heart dysfunction during the development of diabetic cardiomyopathy.

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE **PAGE**

LIST OF TABLES

TABLE **PAGE**

I. REVIEW OF LITERATURE

1. Introduction

Diabetes mellitus is a chronic disorder characterized by impaired metabolism of glucose involving distinct pathogenic mechanisms with hyperglycemia as the common denominator [1]. This disease is associated with deficiency of insulin and/or insulin resistance as a consequence of insulin deficiency or insulin resistance. Hyperglycemia in turn plays a major role in the complications of the disease [2]. In North America diabetes is the fourth major cause of premature disability and mortality. Chronic diabetes increases the risk of cardiac, cerebral and peripheral vascular disease two- to seven-fold [2]. It is associated with blindness, renal disease, neuropathic complication, impaired peripheral artery circulation, coronary artery disease leading to myocardial infarction and cerebral vascular disease resulting in stroke [3]. The Chinese recognized this disease as a syndrome of polyphagia, polyuria, and polydipsia whereas physicians from India noted the sweet taste of the urine and called it "honey urine"[4]; they also suggested the hereditary and environmental factors causing diabetes. However, the name, diabetes was first given by Aretaeus of Cappodocia (200-130 B.C.), which in Greek means 'to run through' or a siphon. Avicenna (980-1037), a Persian physician and author of 'Canon Medicinae', noted the sweet taste of urine in diabetics and found an association between gangrene of limbs and diabetes [5]. In the sixteenth century, Paraclesus (1493-1541), a Swiss physician and pioneer of chemical therapeutics, studied the urine of diabetics and mistook the residue of boiled urine for salt instead of sugar, it was later proven to be glucose which led to a more rational dietary

treatment [3]. In 1859, Claude Bernard, a French physiologist, recognized hyperglycemia as a cardinal feature and the glycogenic effect of the liver. Mering and Minkowski in 1889 demonstrated that pancreatectomy in dogs caused diabetes. A solution to test urine for glucose was introduced by an American physician Staneley Benedict (1884-1936). A Romanian physiologist, Nicholae Paulescu (1869-1931), demonstrated in 1921 that hypoglycemia could be introduced in dogs by injecting pancreatic extract and he named the substance 'pancrein'. Frederick Banting (1891-1941), a Canadian surgeon, and John Macleod (1876-1935), a Physiologist at the University of Toronto, won the Nobel prize in Biochemistery in 1923 for their discovery of insulin in 1921. The prize money was shared with co-workers Charles Best (1899-1978) and James Collip (1892-1965) [6]. The discovery of insulin has totally changed and revolutionized the treatment of diabetes and has saved countless lives worldwide.

2. Classification of Diabetes

Diabetes mellitus can be divided into four subclasses: a) Type I or insulin dependent diabetes, b) Type II or non insulin dependent diabetes, c) Gestational diabetes and d) Other types of diabetes [7].

a. Type I Diabetes

The terms insulin dependent diabetes mellitus and type I diabetes are used synonymously [8, 9]. Patients with this disorder have little or no insulin secretory capacity and depend on exogenous insulin to prevent metabolic decompensation such as ketoacidosis,

and death. Commonly diabetes occurs abruptly over days or weeks in healthy non-obese children or young adults, whereas in older age groups it may have a more gradual onset. In type I diabetes pancreatic beta cells are gradually destroyed by an autoimmune attack and thereafter the insulin secretory capacity is gradually lost. Type I diabetes accounts for about 10% of diabetes in North America.

b. Type II Diabetes

The terms non-insulin diabetes mellitus and type II diabetes are used synonymously [8, 9]; this is the most common form of diabetes, comprising 85-90% of the diabetic population. Affected patients retain some endogenous insulin secretory capacity. Patients do not depend on insulin for immediate survival and rarely develop ketosis. Type II diabetes characteristically appears after the age of 40 years.

c. Gestational diabetes

This is a temporary condition that occurs during pregnancy. It affects about 1 in 20 pregnant women. 40% of these mothers will go on to develop type II diabetes later in life. This type of diabetes is associated with older age, obesity, family history of diabetes. It is also associated with increased risk of macrosomia [2].

d. Other types of diabetes

These include diabetes secondary to or associated with pancreatic disease, hormonal disease, drug or chemical exposure, and certain genetic syndromes. In addition to the presence of specific condition, hyperglycemia is also observed in this type of diabetes.

3. Complications of Diabetes

Complications of diabetes can be divided into two groups: a) microvascular complications, which include retinopathy, neuropathy and nephropathy, b) macrovascular complications, which include atherosclerosis, stroke, myocardial infarction and gangrene. The number of deaths from acute metabolic complications due to diabetes has decreased considerably since the availability of insulin and antibiotics.

The prevalence of coronary artery disease (CAD) is increased among patients with diabetes mellitus (DM) [10]. It is the most common cause of death in non-insulin dependent diabetes mellitus (NIDDM) and it contributes significantly to mortality in insulin dependent DM [11, 12]. An increased incidence of myocardial infarction (MI), angina and sudden death has been observed in patients with DM [13, 14]. Myocardial infarction is the cause of death in about 20% of patients with DM [15]. This is the result of an increased incidence of CAD, particularly in women [16-18]. Complications of MI such as congestive heart failure, cardiogenic shock, arrhythmias and myocardial rupture are also more frequent in DM [16, 19, 20]. Silent MI, which commonly occurs in DM patients, has also been reported to occur without autonomic neuropathy [16, 19, 20]. Silent ischemia is a common feature in patients with DM and CAD may occur in diabetics without autonomic neuropathy [21, 22]. In patients with multivessel coronary disease undergoing multivessel angioplasty, the presence of diabetes predicted procedural failure; major ischemic complication such as restenosis also occurs more frequently [23, 24]. Heart rate (HR) variations with respiration and standing are decreased in diabetics, especially in those with evidence of peripheral or

autonomic neuropathy [25-27]. Defects in parasympathetic innervation, expressed as an increased resting (HR) and a decreased respiratory variation in HR, are more frequently seen in the early stages of DM [28]. On the other hand, defects in sympathetic innervation, seen as a decreased HR rise during standing, are less frequent and occur in the later stages of DM [28]. A prospective study of DM patients with and without autonomic neuropathy revealed a markedly diminished survival and a substantial number of sudden deaths in those with neuropathy [26]. An increased tendency of having ventricular arrhythmias leading to sudden death may be associated with QT prolongation. A recent study showed a high incidence of QT prolongation at rest, mainly after exercise, in diabetics with autonomic neuropathy, [29].

Clinical and pathological reports have documented the existence of a diabetic cardiomyopathy or heart muscle disease independent of coronary atherosclerosis [30, 31]. An increased incidence of congestive heart failure without clinical evidence of CAD or valvular heart disease has also been observed in diabetic patients [32]. Pathological studies have shown myocardial hypertrophy and interstitial fibrosis [31] and have focused on the associated effects of hypertension on the development of diabetic cardiomyopathy [30]. Forensic studies revealed cellular hypertrophy and interstitial and myocytolytic necrosis with replacement fibrosis [33]. Diabetic cardiomyopathy may also occur in the setting of other atherosclerotic CAD which may explain the increase in frequency of congestive heart failure following MI. Systolic time intervals in the preejection period and left ventricular ejection time are frequently abnormal, depicting decrease in contractility and/or a reduction in preload, possibly due to diminished left ventricular compliance [34]. Treatment of

hyperglycemia has been associated with partial normalization of changes in systolic time intervals [35]. Young patients with DM have a high frequency of diastolic dysfunction demonstrated by pulsed Doppler echocardiography, the ratio of peak early to peak late atrial filling velocity is significantly lower in patients with DM [36]. Experimental studies support the view that the diabetic state itself affects ventricular performance. In diabetic rats, left ventricular papillary muscle shows marked slowing and prolongation of contraction with delayed relaxation [37]. With the development of diabetes, myosin ATPase decreases, with a shift in the myosin isozymes from the faster V1 form to the slower V3 form [38]. The slowed relaxation of the diabetic muscle correlates with a reduction in the rate of Ca^{2+} uptake by the sarcoplasmic reticulum (SR) [39]. All of these cardiac alterations in the experimentally induced diabetic animals are reversed by insulin treatment [37]. An important role of altered myocardial calcium metabolism in diabetes is suggested by the beneficial effects of verapamil on hemodynamic function and subcellular biochemistry in diabetic rats [40].

4. Experimental Models of Diabetes

Diabetes both in humans and animals may be provoked by stress, infection or toxins. Certain other manipulations, including pancreatectomy, and lesions of the central nervous system, can also produce diabetes. The use of chemical agents to produce diabetes permits detailed study of the biochemical, hormonal and morphologic events that occur during and after the induction of diabetes. Several classes of agents produce such effects: a) cell

specific toxins which destroy beta cells and cause a primary insulin-deficient state; b) substances which act on the beta cell but do not destroy it; c) agents which increase endogenous insulin requirements, stress the pancreas and secondarily produce diabetes; and d) contra-insulin hormones, as well as anti-insulin antibodies. Two agents which have been most extensively studied and have yielded the vast majority of information pertinent to human diabetes are alloxan and streptozotocin.

Alloxan was first noted to possess diabetogenic activity by Dunn in the course of studies on the effect of uric acid derivatives on the kidney [41]. Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) has a complex chemical structure and it exists in several tautomeric forms. Available evidence indicates that the initial effect of alloxan is mediated through some form of membrane interaction on the surface of the beta cell. Autoradiographic studies have revealed a high affinity of the drug for islet cell membranes. Evidence has also been advanced for membrane permeability changes that could lead to necrosis [42, 43] but a definitive explanation for the highly specific toxicity of alloxan is lacking. The administration of alloxan to a susceptible animal, results in a slight fall in blood glucose followed by mild hyperglycemia within 1 to 4 hours; hypoglycemia occurs probably as a result of the release of stored insulin by dying beta cells. Permanent hyperglycemia is established within 24 to 48 hours of alloxan administration. Histopathologically, reduction of beta cell numbers with sparing of other islet components is noted.

Streptozotocin, a N-nitroso derivative of D-glucosamine, was initially isolated from cultures of *Streptomyces achromogenes*, but subsequently has been synthesized in the

laboratory [44]. Like alloxan, streptozotocin is a relatively selective beta-cytotoxin in certain animal species, causing an initial triphasic glucose response and then permanent diabetes. Cell membrane binding is the likely first step in the pathologic process. In the case of streptozotocin, the alpha anomer of the glucosamine moiety has been shown to render the compound more cytotoxic than the beta anomer, suggesting that the toxicity of the drug is mediated through specific recognition by some receptors on the beta cell [45]. It has further been suggested that the glucose component of streptozotocin enhances its uptake into the beta cell where the cytotoxicity is induced by the nitrosourea moiety [46-48]. Within the beta cell, streptozotocin is believed to reduce the level of nicotine adenine dinucleotide by both decreasing its synthesis and increasing its breakdown [48]. Nicotinamide protects animals against the cytotoxicity of both streptozotocin and alloxan. Histopathologically, beta cell necrosis without insulitis is observed [49, 50]; the effective dose of streptozotocin to induce diabetes may vary from 35 to 75 mg/kg body weight.

5. Regulation of Ca^{2+}-Movements and Heart Dysfunction in Diabetes

Depolarization of cardiomyocytes (normally by an action potential) leads to a voltage dependent opening of L-type Ca^{2+} channels in the membrane, resulting in entry of a small amount of Ca^{2+} [51-59]. A small amount of Ca^{2+} may also enter via the Na^{+}/Ca^{2+} exchange operating in the reverse mode [60, 61]. This small amount of Ca^{2+} triggers a much larger release of Ca^{2+} from the main intracellular store, the SR [51, 62]. When the SR is triggered to release Ca^{2+}, there is a transient rise in the cytoplasmic concentration of Ca^{2+} from a

resting level of 100 nM to a peak between 1 and 2 μM within 20 to 40 msec after depolarization [54, 63]. The rise in Ca^{2+} concentration activates the myofilaments and produces the cardiac contraction. This process of Ca^{2+}-induced Ca^{2+}-release is widely accepted as the major mechanism of SR Ca^{2+}-release in the heart [64-66]. After the initial release of Ca^{2+} there is a period of recovery during which Ca^{2+} is pumped back into the SR by the SR Ca^{2+}-stimulated ATPase [67] and extruded from the cell primarily by the Na^{+}/Ca^{2+} exchanger, which is present in the sarcolemmal membrane in addition to Ca^{2+}-ATPase [68]. Although activation of the sympathetic nervous system has been shown to increase the activities of sarcolemmal L-type Ca^{2+}-channels and Ca^{2+}-pump ATPase, no conclusive information with respect to sarcolemmal Na^{+}/Ca^{2+} exchanger is available. Nonetheless, activation of the sympathetic nervous system is now well known to increase the SR Ca^{2+}-pump and Ca^{2+}-release activities in the heart.

The SR in the cardiac muscle is an important intracellular membrane system which is involved in the relaxation process in the cardiac muscle. When Ca^{2+}-pump ATPase transports Ca^{2+} from the cytosol to the intraluminal side of the sarcoplasmic reticulum the relaxation of cardiac muscle occurs. A phosphoprotein called phospholamban regulates the Ca^{2+}-pump. Phosphorylation of phospholamban takes place at distinct sites by protein kinases such as cAMP-dependent protein kinase, Ca^{2+}-calmodulin-dependent protein kinase, and Ca^{2+}-phospholipid-dependent protein kinase [69]. After phosphorylation of phospholamban, there is an increase in the transport of Ca^{2+} by SR [70-74]. Protein phosphatase activity associated with SR can reverse the stimulatory effect of protein kinases

on Ca^{2+} transport [75]. In fact the intrinsic protein phosphatase is present in cardiac SR membrane, and this phosphatase activity has been shown to dephosphorylate phospholamban and regulate calcium transport [76]. In some studies it has been shown that the phosphatase which dephosphorylates phospholamban in the cardiac muscle had type 2A phosphatase characteristics [77] whereas in other studies there is evidence that type 1 enzyme is the SR-membrane bound phosphatase and is responsible for the dephosphorylation of phospholamban [76]. It has also been reported that the phosphatase responsible for the dephosphorylation of phospholamban is a mixture of both type 1 and 2A enzymes [78]. Thus phosphorylation of SR by different protein kinases and dephosphorylation by different protein phosphatases are considered to regulate the SR Ca^{2+}-uptake and Ca^{2+}-release activities and play a crucial role in processes involved in determining the status of heart function.

Heart dysfunction in streptozotocin-induced chronic diabetes can manifest in the form of reduced heart rate, depressed peak ventricular pressure and depression in the rates of contraction and relaxation of the left ventricle [37, 79-83]. Congestive heart failure occurs in diabetic rats suffering from hypertension [84, 85]. It has been highlighted that heart dysfunction in chronic diabetes is mainly due to subcellular abnormalities in the myocardium [86]. An important role is played by the depressed ATPase activities of contractile proteins in the development of heart dysfunction in diabetes. Remodelling of both regulatory and contractile proteins takes place in diabetic cardiomyopathy and these changes in the diabetic heart are depicted by ATPase activity. The diabetic heart is not able to relax fully as the SR

Ca^{2+}-pump activity is depressed [80, 87]. It has been reported that there is an increase in myocardial Na^{+} and Ca^{2+} content in diabetes [88, 89] and functional abnormalities are caused by intracellular Ca^{2+} overload in the heart [90, 91]. A major role in the development of diabetic cardiomyopathy is played by a change in metabolism of the myocardium, which results from a decrease in the utilization of plasma glucose and an increase in the consumption of free fatty acids. Studies have shown that oxidative processes involving free radicals play an important role in the pathogenesis of diabetic complications [92-96]. It has also been stated that changes such as cardiomyopathy, autonomic neuropathy and perivascular fibrosis may develop as a result of oxidative stress in chronic diabetes [97]. It has shown that treatment of diabetes with antioxidants increases the level of the latter in tissues and improves insulin targeting, and function of the endothelium in addition to decreasing the oxidative load. Several studies demonstrate the role of oxidative stress in diabetes-induced complications [98-103].

6. Phosphorylation-Dephosphorylation System and Cellular Function

Phosphorylation and dephosphorylation of proteins were described by Edmond Fischer and Edwin Krebs over four decades ago and their seminal contributions to research in this area were recognized in 1992 with a Nobel Prize. The process of reversible phosphorylation now is recognized as a universal mechanism for the post-translational control of protein function. All physiological processes are subject to this type of regulation, including, transcription, translation, ion transport, cell structure, motility, mitosis and cell

cycle progression [104]. The dephosphorylation of proteins on their serine, threonine and tyrosine residues is catalyzed by three families of protein phosphatases that regulate numerous intracellular processes. Diversity of structure within a family is generated by targeting the regulatory subunits and domains. Structural studies of these enzymes have revealed that although two families of protein serine/threonine phosphatases are unrelated in sequence, the architecture of their catalytic domains is remarkably similar and distinct from the protein tyrosine phosphatases [105]. Reversible protein phosphorylation is a critical component of the signal transduction mechanisms by which extracellular signals regulate homeostasis and cell growth. Extracellular effectors act by modulating protein kinases and protein phosphatases, which catalyze the opposing activities of protein phosphorylation and dephosphorylation, respectively [106]. Change in the phosphorylation state of proteins on their serine, threonine and tyrosine residues are responsible for disparate protein confirmational and hence functional changes [107], and this, together with its reversibility and scope for signal amplification, probably accounts for the importance of protein phosphorylation in signal transduction. Protein phosphatases (PPs) are structurally and functionally diverse enzymes which are represented by three distinct gene families. Two of these, the PPP and PPM families, dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine. The most abundant protein serine/ threonine phosphatases of the eukaryotes, PP1, PP2A and PP2B, belong to the PPP family whereas PP2C and the related mitochondrial pyruvate dehydrogenase phosphatase are members of the PPM family. Within

each family, although the catalytic domains are highly conserved, suggesting similarities in tertiary structure and catalytic mechanisms, considerable structural and functional diversity of individual protein phosphatases is created as a result of a combination of associated regulatory domains and subunits.

Protein serine/threonine phosphatases of the PPP family play numerous roles in mediating intracellular signalling processes [108, 109]. In addition to PP1, PP2A and PP2B, related novel protein phosphatases have recently been characterized that occur in low abundance and in a tissue and developmental specific manner [110]. PP1 and PP2A are specifically inhibited by a variety of naturally occurring toxins such as okadaic acid, a diarrhetic shellfish poison and strong tumor promotor, and microcystin, a liver toxin produced by blue green algae [111]. Whereas PP2B is only poorly inhibited by the toxins that affect PP1 and PP2A, it was recently defined as the immunosuppressive target of FK506 and cyclosporin in association with their major cellular binding proteins, the cis-trans peptidyl propyl isomerases FKBP12 and cyclophilin, respectively [112]. The structural complexity of PP1 and PP2 holoenzymes in vivo resolves the seemingly paradoxical situation that a relatively small number of protein phosphatase catalytic subunits are responsible for the specific dephosphorylation of a variety of cellular proteins, and that both PP1 and PP2A have been implicated in regulating many diverse cellular functions, including glycogen metabolism, muscle contraction, control of the cell cycle and RNA splicing. As the individual catalytic subunits of PP1 and PP2A catalyze the dephosphorylation of a broad and overlapping range of substrates in vitro, specificity in vivo is generated either by altering

the selectivity of the enzyme towards a particular substrate or by targeting the phosphatase to the subcellular location of its substrates. This is achieved by regulatory or targeting subunits that bind to the phosphatase catalytic subunits [113, 114]. In addition, the regulatory subunits allow the activity of the PPPs to be modulated by reversible protein phosphorylation and second messengers [113, 114].

a. Protein Phosphatase 1

The catalytic subunit of PP1, a 37 kDa protein, has been crystallized in the presence of the phosphatase inhibitor cystin [115]. In rat, cDNA cloning revealed the existence of at least four isoforms, termed α, γ1, γ2 and δ [116]; interestingly, the γ1 and γ2 isoforms are produced by alternative splicing of the same primary transcript. The native structure of PP1 is a 1:1 complex between the catalytic and a number of different regulatory subunits [113]. The role of these regulatory subunits is to target the catalytic subunit towards specific subcellular locations and to increase activity towards particular substrates. The holoenzymes are named according to their apparent subcellular location [117]. For example, the glycogen bound PP1 holoenzyme (PP1G) is a heterodimer of PP1c and a glycogen binding subunit (G-subunit) [118]. The G-subunit anchors the phosphatase to glycogen and increases its activity towards the glycogen bound substrates: glycogen synthase and glycogen phosphorylase. These substrates are activated and inactivated respectively by dephosphorylation [113]. It is now clear that PP1 is involved in many different cellular processes, such as glycogen metabolism, calcium transport, muscle contraction as well as protein synthesis and intracellular transport [117, 119]. In some cellular processes the PP1

substrates including glycogen synthase, glycogen phosphorylase, myosin light chain, phospholamban and ribosomal protein S6 have been identified [119, 120]. Recent evidence from the study of cell cycle mutants has revealed that PP1 also plays an important role in the regulation of mitosis. Further evidence for the role of PP1 in mitosis was obtained in a study of drosophila mutants [121, 122].

b. Protein Phosphatase 2A

Several trimeric holoenzyme forms of PP2A have been extensively characterized. The core of these structures consists of a 36 kDa catalytic subunit complexed with a regulatory subunit of 65 kDa [123, 124]. In vivo, PP2A is probably only present as a trimer, but this remains a controversial issue because the core dimer has been purified from many different tissues; this suggests the possibility of subunit rearrangements where the variable subunits are able to associate and dissociate from the core dimer. The exact function of the regulatory subunits is not yet completely resolved, but they probably influence substrate specificity and/or subcellular localization. [125, 126]. Molecular cloning reveals the existence of several isoforms of each subunit [121, 127]. Most PP2A subunits appear to be ubiquitously expressed; PR 55β and γ are mainly limited to neuronal tissues [123] whereas PR 72 transcripts are found in muscle tissue only [125].

Through the use of okadaic acid and related inhibitors, it was possible to specifically inhibit PP2A activity in cell free extracts. Although one cannot exclude the possibility that PP2A like enzymes are affected, this approach, often in combination with purified PP2A preparations, has been used to identify potential PP2A substrates in a wide variety of cellular

processes such as metabolism, muscle contraction, synaptic transmission, signal transduction, RNA splicing and cell cycle progression [109, 124]

c. Protein Phosphatase 2B and Protein Phosphatase 2C

PP2B or calcineurin was first identified as a major calmodulin binding protein from brain where it accounts for up to 1% of the total protein [128]; however later it was shown to display serine/threonine protein phosphatase activity. PP2B is a heterodimer of calcineurin A (molecular mass 60 kDa) and calcineurin B (19 kDa). Calcineurin A is the catalytic subunit and binds to calmodulin whereas calcineurin B is the regulatory, Ca^{2+} binding subunit. Calcineurin B is a member of the family of calcium binding proteins and was shown to contain four calcium binding loops and 35% sequence identity to calmodulin [108]. On the other hand, PP2C was originally identified as a Mg^{2+} dependent protein phosphatase this enzyme is monomeric with a molecular mass of 43-48 kDa [129]. Two major isoforms (α, β) of PP2C have been cloned. Further analysis revealed the existence of subtypes for both isoforms. Three α subtypes have been identified, with $\alpha 1$ being the most dominant species. Two forms of β isoforms appear to be the result of alternative splicing since they differ only at their C-terminus. Whereas $\beta 1$ is ubiquitously expressed, $\beta 2$ transcript is found in brain and heart [130, 131]. PP2C may catalyze the dephosphorylation of autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II the cerebellum [132].

d. Substrate Specificity and Regulation of Protein Phosphatases

The study of the specificity of protein serine/threonine phosphatases in vitro has shown that these enzymes have broad substrate specificities [118]. Regions of protein

substrates other than those immediately adjacent to the phosphorylation site are likely to be important in substrate binding. The protein serine / threonine phosphatases may fall into two categories with regard to their substrate specificities [133]. Type 2A protein like enzymes utilize at least some determinants that are contained in the primary sequence surrounding the phosphorylation site. The other category is enzymes like PP1 and PP2B that have a clear requirement for structural determinants outside the immediate vicinity of the phosphorylation site. There are six regions within the primary structure of these enzymes that are highly conserved between the PP1 / PP2A / PP2B family. The protein serine / threonine phosphatases contain a short stretch of homology with mammalian purple acid phosphatases [134].

The extent of phosphorylation of cellular proteins is controlled by the relative activities of protein kinases and protein phosphatases [135]. The activity of PP1 is regulated in vitro by interaction of the catalytic subunit with endogenous regulatory subunits and inhibitory proteins. Three heat stable inhibitor proteins have been identified in many tissues [124]. The activity of the PP1 catalytic subunit is also controlled by interaction with the G subunit, which serves to stimulate its activity and allow association with the protein glycogen complex in skeletal muscle. In addition, the activity of PP1 toward glycogen synthase in the liver appears to be allosterically regulated by phosphorylase a. On the other hand, type 2A protein phosphatase is regulated by the differential expression of regulatory proteins that control enzyme specificity and activity by formation of oligomeric complexes [136]. Protein phosphatase 2B is comprised of a complex between the catalytic A subunit and a

Ca^{2+}-binding B subunit. The B subunit binds 4 mol Ca^{2+} and undergoes a Ca^{2+}-induced confirmational change that results in the stimulation of the A subunit phosphatase activity; however, the B subunit is not required for activity [137]. In the presence of physiological concentrations of Ca^{2+}, calmodulin forms a 1:1 molar complex with PP2B and stimulates protein phosphatase activity [119]. The interaction of the Ca^{2+}/calmodulin complex with PP2B suggests that PP2B is primarily a calmodulin-regulated protein phosphatase [124].

e. Inhibitors of Protein Phosphatases

The discovery of several low-molecular-mass protein phosphatase inhibitors, has advanced our knowledge of the function of protein phosphatases [138]. The most widely used agent is okadaic acid. This polyether fatty acid is produced by marine dinoflagellates and causes diarrhoetic shellfish poisoning. It is a potent inhibitor of PP2A and PP1, but is a much less efficient inhibitor of PP2B [129, 139]. Thus, okadaic acid in cell free extracts is a valuable tool used to distinguish between different phosphatases [108, 120]. These are derivatives of okadaic acid which increase the phosphorylation state of a number of proteins [140] including vimentin and the heat shock protein [141, 142]. Okadaic acid also increases the phosphorylation state of the epidermal growth factor receptor [143], histone H3 [144], a progesterone receptor [145], the α-subunit of the inhibitory nucleotide binding protein Gi [146] and cardiac regulatory proteins [147]. The physiological relevance for the action of phosphatase inhibitors can be inferred from their use in the pathogenesis of disease. Deregulation of protein phosphatases has also been implicated in insulin resistance associated with diabetes mellitus and other metabolic disorders [148]. Because of the in

effect in dopaminergic pathways in the brain, phosphatase inhibitors have been indicated in Parkinson's disease [149]. There appears to be a strong link between PP2A inhibition and cancer [148]. It is not certain to what extent the failure of hormones to control protein phosphatase activity is due to errors in the function of endogenous inhibitors. In fact it was suspected that the tumor promoting activity of okadaic acid was due to its regulation of the same pathway that is affected by the well-established phorbol ester tumor promoters, namely the protein kinase C (PKC) pathway [120, 150, 151]. Since the activation of PKC by phorbol esters initiated a kinase cascade that lead to the increased phosphorylation/activation of various downstream components of this signal transduction pathway, okadaic acid, is likely to generate the same effect by increasing the activity of this pathway through the inhibition of phosphatases that dephosphorylate/ inactivate the same components [152]. It was observed that okadaic acid, calyculin A, and cantharidin were able to prevent apoptosis in short term, but not in long term experiments [153]. Similarly, the level of phosphorylation of the retinoblastoma tumor suppressor protein, an important regulator of cell cycle progression, is affected differentially by okadaic acid, depending on the time of incubation and concentration of the drug [154-157]. The existence of toxins and viral proteins that target serine/threonine phosphatases reveals the importance of reversible regulation of endogenous phosphatase inhibitors for proper functioning of phosphatases in cells [148]. Although protein phosphatase 1 and protein phosphatase 2A are abundantly expressed in the heart and skeletal muscle, no information regarding the status of these enzymes during the development of diabetes is available in the literature. Accordingly, it is proposed to

investigate changes in the protein phosphatase activities in the heart and skeletal muscle during the development of streptozotocin-induced diabetic cardiomyopathy in rats.

II. MATERIAL AND METHODS

1. Experimental Model

Male Sprague Dawley rats weighing between 200 and 250 g were made diabetic with a single injection of streptozotocin monohydrate at a dose of 65 mg/kg into the tail vein; this dose of streptozotocin yields 100% diabetogenesis. Rats were anesthetized with halothane 2% prior to injection of streptozotocin. Streptozotocin was dissolved in a 0.05 M citrate buffered (pH 4.5) saline solution just prior to injection. Streptozotocin has been reported to cause non-specific kidney damage, therefore 5 to 10 ml of 0.9% NaCl was given intraperitoneally immediately following streptozotocin administration, as suggested by Heimberg et al. [158, 159] in order to diminish kidney damage [160, 161]. Since rats injected with streptozotocin undergo a transient and dangerously fatal hypoglycemic period, a 50% dextrose-saline solution was administered subcutaneously within 12-24 hours after administration of streptozotocin to minimize and prevent mortality. These precautions resulted in a mortality rate of less than 2% in this experimental model. Three days after streptozotocin injection, rats displaying glycosuria (>2%) and plasma glucose elevation (>300 mg/ 100 ml) were used as the diabetic group. Control rats received an injection of only citrate buffered saline solution (without streptozotocin). Throughout the course of experiment, food and water were provided *ad libitum*. Diabetic and control rats were used at 1, 2, 4 and 8 weeks following the injection of streptozotocin or vehicle, respectively.

Four weeks after streptozotocin injection, a random group of diabetic animals were given daily a subcutaneous injection of ultralente insulin (Eli Lilly Canada) for a period of

4 weeks. The dose of insulin was adjusted to achieve blood glucose levels in the range of 100-200 mg/100 ml. The approximate dose of insulin used was 3 U/day. All rats were killed by decapitation. A drop of trunk blood was applied on a glucose monitoring stick and blood glucose determined using a glucose monitoring machine (Elite, Bayer Inc., Toronto). Some trunk blood was collected at the time of death in heparinized tubes to avoid coagulation. Plasma was prepared from the blood samples upon centrifugation at 3000 × g and was stored at -20°C for RIA analysis of insulin (Linco-rat-insulin RIA kit). Hearts were immediately removed and the ventricular tissue was separated from atria, connective tissue as well as major blood vessels and weighed. Ventricular tissue was washed in 10 mM EDTA solution to remove any adhering blood and then placed in liquid nitrogen and later stored at -70°C. The hind leg skeletal muscle was also used in some experiments.

2. Hemodynamic Assessment of Animals

The animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (9.0/0.9 mg/kg), the trachea was intubated to maintain adequate ventilation, the right carotid artery was exposed, and a microtip transducer (Model SPR-249, Millar) was introduced through proximal arteriotomy [162, 163]. The catheter was advanced carefully through the lumen of the carotid artery until the tip of the transducer entered the left ventricle. The catheter pressure transducer was secured with the help of a silk ligature around the artery and readings were recorded on a computer system using Acqknowledge 3.1 Software. Left ventricular rate of contraction (+dP/dt) and rate of relaxation (-dP/dt) were recorded after 10-

15 minutes of stabilization.

3. Preparation of Tissue Extracts for Determination of Enzyme Activities

Tissue extracts were obtained by homogenizing samples with a Brinkmann polytron. One hundred mg of cardiac tissue or skeletal muscle was taken in 10 volumes of 250 mM sucrose, 4 mM EDTA, 30 mM β-mercaptoethonol and 20 mM Tris-HCl (pH 8.3, at 4°C) then they were put in a test tube corresponding to the size of the polytron and minced with scissors before giving two 30 second bursts with the polytron in the cold room at 4°C with one minute interval between bursts [164]. The homogenates were then centrifuged at 12000 × g for 15 minutes. The supernatants were subjected to sephadex G-50 gel filtration to remove low molecular weight protein phosphatase inhibitors. For this purpose 500 µl of supernatant was loaded on a Sephadex column (4.5 ml bed) and eluted with a buffer (50 mM Tris-HCl pH 7.7, at 4°C, 0.1 mM EDTA, 0.1% Brij 35 and 30 mM β-mercaptoethanol). The eluate of the column was collected for a duration of six minutes. The protein concentration was measured by the Lowry's method and the aliquotes were stored at -70°C for protein phosphatase assay.

4. Protein Phosphatase Assay for PP1 and PP2A Activities

Assay diluation buffer 1 contained MOPS 200 mM, $MnCl_2$ 1 mM, $MgCl_2$ 10 mM, EGTA 20 mM, NaCl 1.5 M dissolved in distilled water; 10% glycerol, 0.1 mg/ml bovine serum albumin and 60 mM β-mercaptoethanol were added freshly on the day of experiment.

Assay dilution buffer 2 contained MOPS 200 mM, β-mercaptoethanol 60 mM, NaCl 1M, bovine serum albumin 1 mg/ml were dissolved in distilled water. β-mercaptoethanol and bovine serum albumin were added freshly on the day of experiment. 2.5 µl of assay dilution buffer 1 for measuring PP1 activity and 2.5 µl assay dilution buffer 2 for measuring PP2A activity were added in a microtiter well. Distilled water in the amount of 6.25 µl was added in the assay well and 12.5 µl was added in the blank. After that 10 µl of eluted sample was added in assay well as well as blank. The reaction was initiated by adding 6.25 µl of substrate in the assay well but not in the blank. Distilled water was added to make the total volume in the well 25 µl. The microtiter plate was than incubated for 5 minutes at 30°C in a shaker incubator. The enzyme reaction was terminated by the addition of 100 µl malachite green solution provided with the kit (Upstate Biotechnology Inc. Canada). The microtiter plate was then kept at room temperature for 15 minutes to allow color development. After 15 minutes the absorbance of each well was measured in a microtiter plate reader at 620-660 nm. Absorbance of blank solution was subtracted from enzyme reaction values. Using the Upstate Biotechnology kit for protein phosphatase assay, 0.1 U of protein phosphatase 1 or 2A released 46.7 pmole Pi/min, which corresponded to an absorbance of 0.106 above backround. In some experiments the effect of okadaic acid 0.01 nm to 1 µM on phosphatase activities were also examined.

5. Statistical Analysis

Results are expressed as means ± SE of 6 experiments. Statistical analysis was carried out by Students t-test, and a P level < 0.05 was taken to reflect a significant difference between control and experimental groups.

III. RESULTS

1. General Characteristics and Hemodynamic Changes in Experimental Rats

The data in Table 1 indicates that the diabetic rats had significantly lower body weights and ventricular weights 8 weeks after streptozotocin administration. In addition, the diabetic animals exhibited a significantly higher ventricular to body weight ratio. The presence of diabetes in these experimental animals, was confirmed by markedly elevated plasma glucose levels and severely depressed plasma insulin levels. Daily injections of insulin to the 4 week diabetic rats for 4 weeks partially normalized the plasma glucose as well as ventricular/body weight ratio and fully normalized the plasma insulin level. The hemodynamic assessment revealed significant depressions in left ventricular rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) in the diabetic rats compared to the control groups. These hemodynamic parameters were partially normalized after insulin administration.

2. Cardiac Protein Phosphatase 1 and 2A Activities in Experimental Rats

The data in Table 2 indicate that the diabetic rats had significantly higher protein phosphatase 1 and 2A activities 8 weeks after streptozotocin administration; however, the increase for protein phosphatase 1 activity was 52% whereas that for protein phosphatase 2A activity was 30%. The protein concentrations of the tissue extracts from control and diabetic heart were not different from each other. Furthermore, the phosphatase activities in the diabetic heart were also higher than those in the control group when the enzyme activities

Table 1. General characteristics and hemodynamic parameters of control, diabetic and insulin-treated diabetic animals 8 weeks after injecting streptozotocin

	Control	Diabetic	Diabetic+Insulin
Body wt. (g)	542 ± 21.5	312 ± 21*	354 ± 15
Ventricular wt. (mg)	998 ± 85	833 ± 40*	882 ± 42#
Ventricular/body wt. ratio (mg/g)	1.84 ± 0.05	2.66 ± 0.01*	2.49 ± 0.06#
Plasma glucose (mg/dl)	165 ± 8.2	452 ± 10.2*	203 ± 7.0#
Plasma insulin (μU/ml)	30.1 ± 2.5	11.9 ± 0.5*	36.2 ± 0.8#
+dP/dt (mm Hg/sec)	5701 ± 244	3722 ± 183*	4639 ± 266#
-dP/dt (mm Hg/sec)	5501 ± 320	3356 ± 118*	4482 ± 253#

Values are mean ± SE of 6 animals in each group. * Significantly different from control ($P < 0.05$); # Significantly different from diabetic ($P < 0.05$)

Table 2. Protein phosphatase activities in ventricular tissue extracts from control, diabetic and insulin-treated diabetic animals 8 weeks after injecting streptozotocin.

	Protein concentration (µg/mg heart)	Phosphatase activities (Pi µmol/min/mg protein)		Phosphatase activities (Pi µmol/min/g heart tissue)	
		PP1	PP2A	PP1	PP2A
Control	57.67 ± 1.63	114.91 ± 4.7	146.54 ± 5.5	1217 ± 37	1483 ± 65
Diabetic	56.97 ± 0.64	189.27 ± 8.1*	192.73 ± 5.0*	1618 ± 75*	1725 ± 117*
Insulin treated-diabetic	56.19 ± 5.78	152.62 ± 6.2#	168.65 ± 7.6#	1436 ± 119#	1593 ± 39#

Values are mean ± SE of 6 hearts in each group. * Significantly different from control ($P < 0.05$); # Significantly different from diabetic ($P < 0.05$).

were expressed as per gram heart tissue. Daily injections of insulin to the 4 week diabetic rats for 4 weeks partially normalized the protein phosphatase 1 and 2A activities.

To test if changes in protein phosphatase activities in diabetic rats were limited to the heart, protein phosphatase activities were also measured in the skeletal muscle. The data in Table 3 indicate that the diabetic rats had significantly higher protein phosphatase 1 and 2A activities in skeletal muscle; the increase in protein phosphatase 1 activity was 48% whereas that in phosphatase 2A activity was 38%. Daily injections of insulin to the 4 week diabetic rats for 4 weeks partially normalized the protein phosphatase 1 and 2A activities.

3. Inhibition of Cardiac Protein Phosphatase 1 and 2A Activities by Okadaic Acid

In order to examine if the increase in phosphatase activities in the diabetic heart are due to some changes in the characteristics of the enzymes, the effect of different concentrations of okadaic acid was studied. From Figures 1 and 2, it can be seen that increasing the concentration of okadaic acid decreased the protein phosphatase 1 and protein phosphatase 2A activities in both groups. Cardiac protein phosphatase type 1 activity in control and diabetic animals was completely inhibited at 20nM concentration of okadaic acid whereas 100 nM concentration of okadaic acid was required to completely inhibit protein phosphatase type 2A activity in both the groups. In addition the IC_{50} of okadaic acid for protein phosphatase type 2A activity shifted from 1.89 nM in controls to 5.11nM in the diabetic group whereas for protein phosphatase type 1 activity the IC_{50} of okadaic acid was 6.73nM for both the groups.

Table 3. Protein phosphatase activities in skeletal muscle tissue extracts from control, diabetic and insulin-treated diabetic animals 8 weeks after injecting streptozotocin.

	Protein Concentration (µg/mg heart)	Phosphatase activities (Pi µmol/min/mg protein)		Phosphatase activities (Pi µmol/min/g heart tissue)	
		PP1	PP2A	PP1	PP2A
Control	49.16 ± 1.63	50.97 ± 2.1	50.11 ± 1.9	434 ± 17	427 ± 16
Diabetic	48.39 ± 0.64	75.43 ± 3.2*	69.15 ± 2.9*	646 ± 16*	555 ± 17*
Insulin treated-diabetic	49.22 ± 3.78	43.26 ± 1.7#	42.62 ± 1.5#	390 ± 15#	389 ± 13#

Values are mean ± SE of 6 samples in each group. *Significantly different from control ($P < 0.05$); #Significantly different from diabetic ($p < 0.05$).

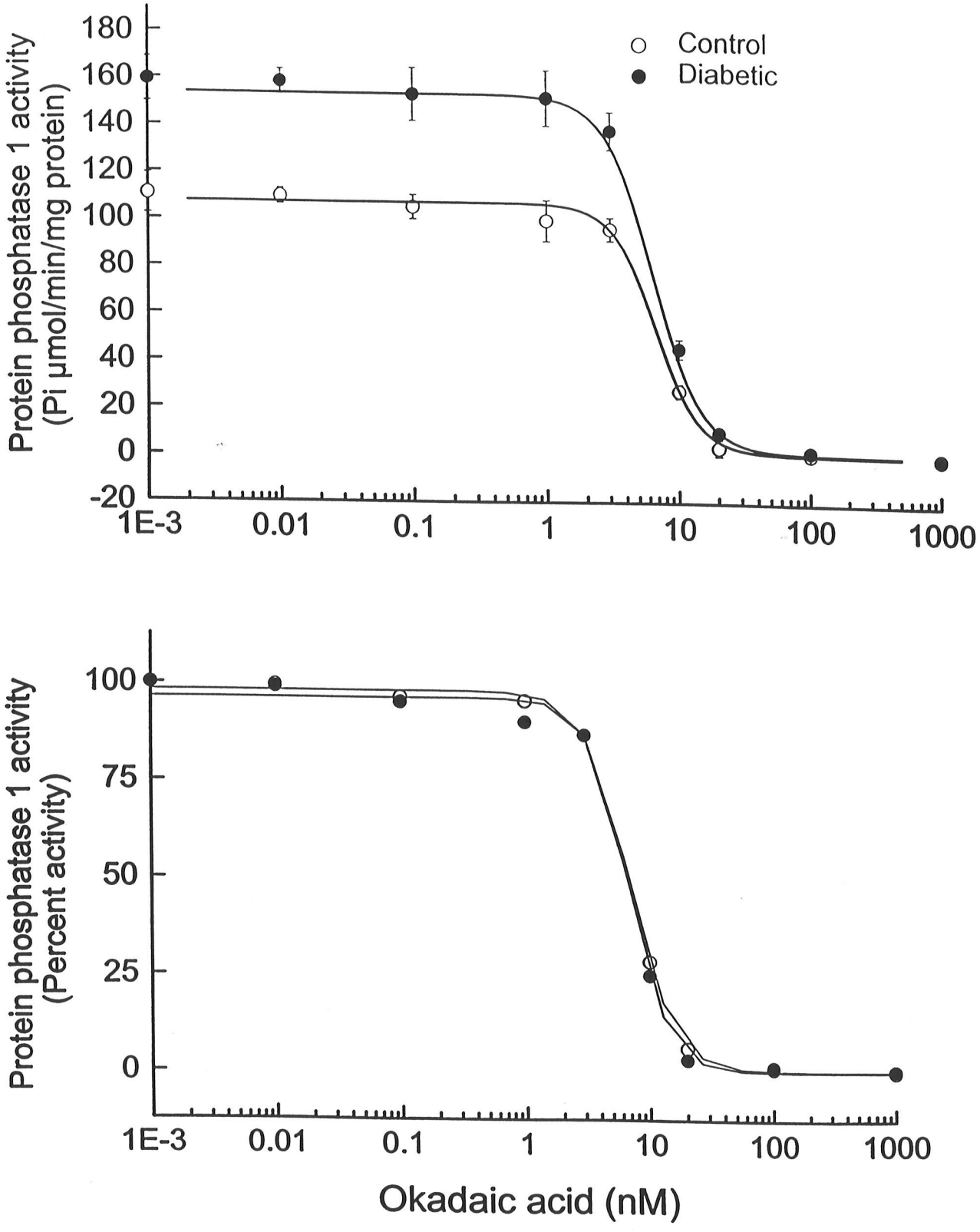

Figure 1: **Inhibition of protein phosphatase 1 activity by okadaic acid.**

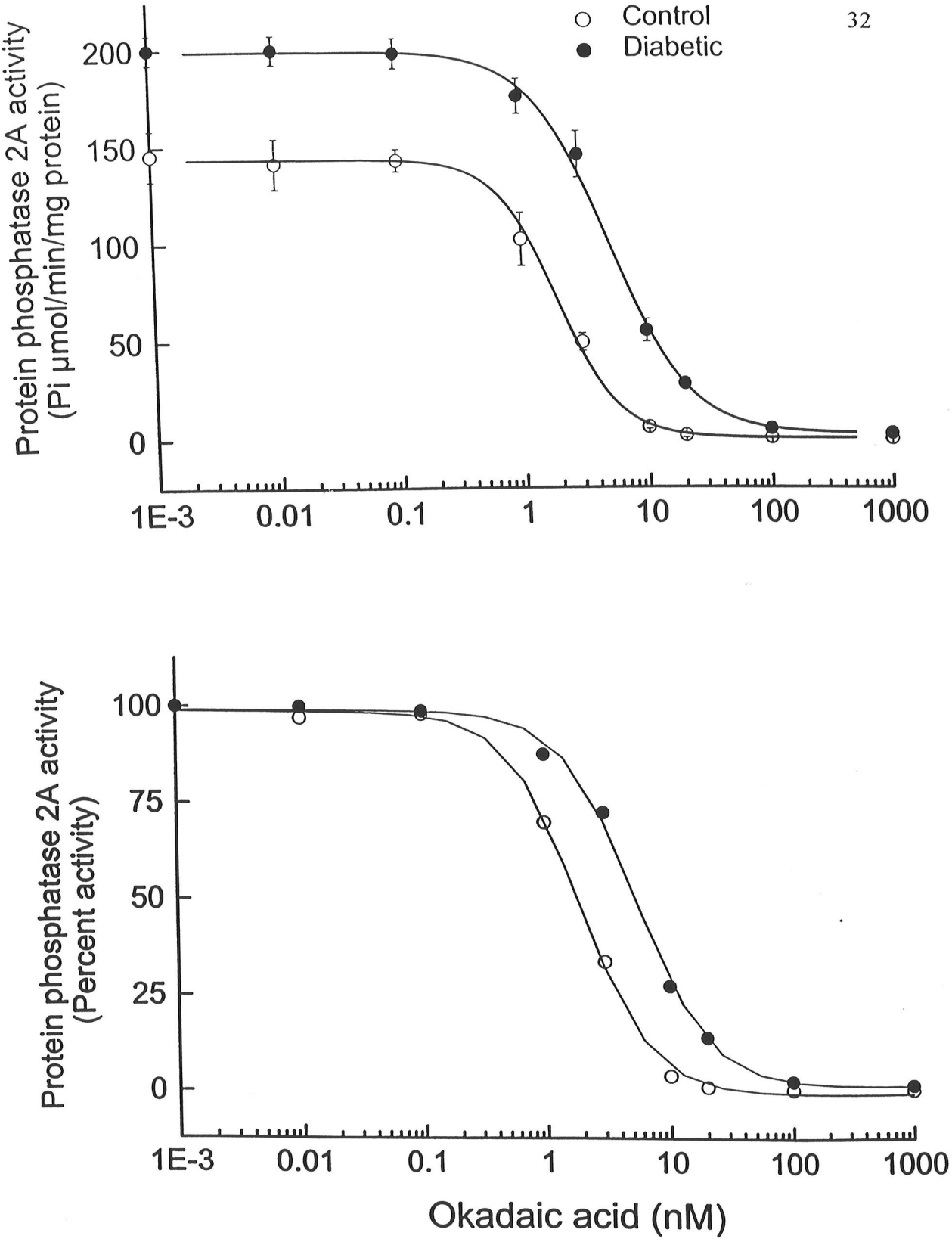

Figure 2: **Inhibition of protein phosphatase 2A activity by okadaic acid.**

4. Relationship Between Changes in Cardiac Function and Cardiac Protein Phosphatase 1 and 2A Activities

In order to examine the relationship between changes in cardiac function and cardiac protein phosphatase 1 and 2A activities, a time course study was undertaken to measure the changes in phosphatase activity at 1, 2, 3 and 4 weeks. The data in Table 4 indicate a significantly higher protein phosphatase 1 and protein phosphatase 2A activities in cardiac tissue at 1, 2, 3 and 4 weeks after administration of streptozotocin. The increase in protein phosphatase 1 activity was maximal (67%) in the second week and then dropped to 44% in the fourth week. Whereas protein phosphatase 2A activity showed maximal increase (75%) in the first week, and then depressed to 36% in the fourth week. The results shown in Table 4 also indicate that there was a progressive decrease in both +dP/dt and -dP/dt upon inducing diabetes; a significant decrease in -dP/dt was evident at 2 weeks whereas that in +dP/dt was evident at 3 weeks of injecting streptozotocin.

Table 4. Contractile and protein phosphatase activities of control, 1, 2, 3 and 4 week diabetic rats

	Contractile activities (mm Hg/sec)		Phosphatase activities (Pi µmol/min/mg protein)	
	+dP/dt	-dP/dt	PP1	PP2A
Control	6052 ± 217	5840 ± 243	114.91 ± 4.7	146.54 ± 5.5
1 week	5860 ± 248	5614 ± 208	173.14 ± 12.5*	255.72 ± 15.1*
2 weeks	5476 ± 225	4618 ± 174*	192.10 ± 5.0*	203.64 ± 16.9*
3 weeks	4836 ± 180*	4272 ± 158*	180.0 ± 3.0*	201.10 ± 9.2*
4weeks	4208 ± 164*	3854 ± 142*	165.0 ± 7.1*	199.35 ± 6.1*

Values are mean ± SE of 4 experiments in each group. *Significantly different from control ($P < 0.05$).

IV. DISCUSSION

Hyperglycemia plays a major role in the development of diabetic complications. It is quite possible that protein phosphatase 1 and 2A are being activated since they participate in the regulation of various subcellular organelles in the myocardium including regulation of calcium transport associated with cardiac sarcoplasmic reticulum [75]. Although the exact mechanism of protein phosphatase 1 and 2A activation in diabetic heart is poorly understood, it has been documented that the phosphatase activities are increased in cardiac dysfunction [165]. In this study, we demonstrated a significant increase in PP1 and PP2A activities in diabetic rat hearts. Diabetes was induced by an intravenous injection of streptozotocin. The exact mechanism by which there is an increase in PP1 and PP2A activities is still not clear.

An important role is played by the depressed ATPase activities of contractile proteins in the development of heart dysfunction in diabetic rats. Remodeling of both regulatory and contractile proteins takes place in diabetic cardiomyopathy. The heart is not able to relax fully due to depressed SR calcium pump activity [80]. It is possible that the observed increase in phosphatase activity may play an important role in the cardiac dysfunction in diabetes.

Phosphatases can be classified into type 1, 2A, 2B and 2C [119]. In the present study only protein phosphatase 1 and 2A activities were studied. Separation by gel filtration (Sephadex G-50) ascertained that both PP1 and PP2A activities are present in the rat heart. It is functionally important that phosphatases should be inhibited as it leads to an increase in phosphorylation of the regulatory proteins in heart, which in turn leads to an increased

force of contraction [147]. The main finding of our study is that there is an increase in protein phosphatase 1 and 2A activities in streptozotocin induced diabetic rat hearts. It may be possible that the increased phosphatase activity is due to increased amounts of phosphatases. Another explanation is that there is a reduction in the level of phosphatase inhibitor 1 [119, 166], which may also lead to an increased phosphatase activity. Studies in other laboratories indicate that nearly all the type 2A activity in the heart muscle is in soluble fraction, whereas type 1 activity is mainly found in cellular organelles [77, 167, 168]. Our findings are in contrast to other studies, where phosphatase activities were decreased in heart and liver [169]. Several studies in other laboratories demonstrate that there was no significant change in the activities of protein phosphatases 1 and 2A in heart and skeletal muscles of diabetic animals [164, 170]. An increased activity of type 1 and 2A phosphatase in the heart may lead to dephosphorylation of phospholamban, which shows up as a decreased uptake of calcium into the SR [171, 172].

Another finding in our study is that okadaic acid is a potent inhibitor of PP1 and PP2A activities. This finding suggests that okadaic acid inhibits the process of dephosphorylation and stimulates the cardiac muscle, which results in a net increase in phosphorylation of proteins, which in turn increases the probability of calcium channels to open [173-175].

The alterations in cardiac and skeletal muscle PP1 and PP2A activities, observed in this study were partially normalized by in vivo treatment of diabetic rats with insulin. It remains unclear whether increased phosphatase activity is the cause of diabetic cardiac dysfunction. However it may contribute to alterations in cardiac contraction and relaxation.

As time-course changes in diabetic heart revealed that the phosphatase activity was increased at 1 week whereas -dP/dt and +dP/dt were depressed at 2 and 3 weeks after the induction of diabetes, it appears that changes in cardiac phosphatase activity may affect the contractile function of the heart.

V. CONCLUSIONS

1. Streptozotocin induced diabetic rats displayed elevated plasma glucose levels and depressed plasma insulin levels, consistent with Type-I (insulin-dependent diabetes mellitus). Decrease in body weight and ventricular weight resulted in an elevated ventricular to body weight ratio. These changes were normalized after insulin treatment (3 U/day; s.c) for 4 weeks.
2. Hemodynamic parameters revealed a decrease in the left ventricular rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) in the diabetic animals as compared to control values. These changes were normalized after insulin treatment for 4 weeks.
3. Cardiac protein phosphatase 1 and protein phosphatase 2A activities in the diabetic animals were markedly increased at 1, 2, 4 and 8 weeks in comparison to control values. In addition there was also a significant increase in protein phosphatase 1 and protein phosphatase 2A activities in the skeletal muscle in comparison to control values. These changes were partially normalized after insulin treatment for 4 weeks.
4. Cardiac protein phosphatase 1 and protein phosphatase 2A activities in control and diabetic animals were completely inhibited at 20 nM and 100 nM concentration of okadaic acid. In addition there was a shift in the IC_{50} value of okadaic acid for protein phosphatase 2A activity from 1.89 nM in controls to 5.11 nM in the diabetic group.
5. These results regarding increased protein phosphatase type 1 and 2A activities indicate enhanced dephosphorylation of subcellular proteins in the diabetic heart and

may contribute towards the development of cardiac dysfunction in the development of diabetic cardiomyopathy. The results regarding inhibition of protein phosphatase type 1 and protein phosphatase type 2A activities by okadaic acid indicate that okadaic acid is a potent inhibitor of these enzymes and suggests that okadaic acid inhibits the process of dephosphorylation and diabetes changes the characteristics of this enzyme.

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