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# Elevated levels of protein phosphatase 1 and phosphatase 2A may contribute to cardiac dysfunction in diabetes

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**Abstract**

Although protein phosphorylation and dephosphorylation are known to regulate the activities of different enzymes, sufficient information on the role of dephosphorylation in cardiac function is not available. Since protein phosphatases mediate dephosphorylation, it is possible that cardiac dysfunction induced by diabetes may be due to alterations in the activities of these enzymes. We therefore determined cardiac protein phosphatase activity as well as protein contents of phosphatase 1 and phosphatase 2A in diabetic animals. For this purpose, rats were made diabetic by administering a single intravenous injection of streptozotocin (65 mg/kg body weight) and hearts were examined after 1, 2, 3, 4 and 8 weeks. Some of the 4-week diabetic animals received subcutaneous injections of insulin (3 U/day) for a further period of 4 weeks. Cardiac dysfunction was evident after 2 weeks of inducing diabetes and deteriorated further with time. A significant increase in protein phosphatase activity appeared after 1 week and persisted until 8 weeks. Increased protein phosphatase activity in the diabetic heart was associated with a corresponding increase in the protein contents of both phosphatase 1 and phosphatase 2A. Insulin treatment partly prevented the changes observed in diabetic animals. The results suggest that increased protein phosphatase activities and subsequent enhanced protein dephosphorylation may play a role in diabetes-induced cardiac dysfunction.


*Keywords:* Diabetes; Protein dephosphorylation; Protein phosphatase; Diabetic heart; Cardiac dysfunction

## 1. Introduction

Diabetes has been reported to induce cardiac dysfunction [1–4], alterations in the activities of cardiac $Ca^{2+}$-cycling proteins such as the sarcolemmal $Na^{+}$–$Ca^{2+}$-exchanger and L-type $Ca^{2+}$-channel [3,5,6] as well as the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase (SERCA2a), phospholamban and ryanodine receptor (RyR) [1,2,4,7]. Furthermore, changes in contractile and regulatory proteins such as myofibrillar ATPase, troponin I and myosin light-chain have been shown to occur in the diabetic heart [8–10]. It is pointed out that the function of all these specialized proteins present on sarcolemma, SR and myofibrils are modulated by regulatory mechanisms such as protein phosphorylation and dephosphorylation where protein kinases catalyze phosphorylation reactions and protein phosphatases mediate dephosphorylation reactions [11].

There is evidence to suggest abnormalities in cardiac protein phosphorylation due to changes in the activities of SR-associated cyclic AMP dependent protein kinase (PKA) and calcium calmodulin-dependent protein kinase (CaMK) as well as cytosolic and particulate protein kinase C in the diabetic heart [12,13]. However, very little information regarding alterations in protein phosphatases, which determine the status of cardiac protein dephosphorylation is available in the diabetic heart. This study was therefore undertaken to examine changes in the protein phosphatase activity during the development of cardiac dysfunction in a streptozotocin-induced model of diabetes in rats [1–10]. Since protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) constitute more than 90% of the total phosphatases in the heart [14,15], this study also measured PP1 and PP2A levels in the diabetic heart. The effect of insulin in reversing the diabetes-induced changes in contractile function, protein phosphatase activity, as well as PP1 and PP2A levels was also examined.

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## 2. Materials and methods

### 2.1. Experimental model

The experimental model employed in this study is similar to that used previously for establishing the presence of diabetic cardiomyopathy as indicated by alterations in cardiac function, metabolism, subcellular organelles and ultrastructure [1–10]. Male Sprague–Dawley rats weighing 200–250 g were randomly separated into control and experimental groups. The experimental animals received an intravenous injection of 0.1 M citrate-buffered streptozotocin (pH 4.5) at a dosage of 65 mg/kg body weight. Control animals received a similar injection of the vehicle alone. These animals were maintained on normal rat chow and water ad libitum and then killed by decapitation after 1, 2, 3, and 4 weeks. Experimental animals displaying glycosuria (>2%) and plasma glucose elevation (>300 mg/100 ml) were considered as the diabetic group. In another set of experiments, a random group of diabetic animals 4 weeks after streptozotocin injection, were administered subcutaneous injections of human insulin ultralente (Eli Lilly Canada, Toronto, ON) for a further period of 4 weeks; the insulin dose was 3 U/day. Plasma glucose and insulin levels were determined by using the Sigma diagnostic kit (Sigma-Aldrich Canada, Oakville, ON) and Linco rat insulin RIA kit (Linco Research Inc., St. Charles, MO), respectively. The in vivo cardiac function was assessed according to the method described earlier [2,13]. Briefly, the right carotid artery was isolated in anaesthetized animals and an ultra-miniature catheter connected to a pressure transducer was inserted into the left ventricle. The rate of pressure development (+dP/dt) and rate of pressure fall (−dP/dt) was recorded by using a computer program, Biopac data acquisition system (Biopac System Inc., Goleta, CA).

### 2.2. Preparation of tissue extracts

Cardiac and skeletal muscle tissue extracts for protein phosphatase activity determination was isolated by the method described by Foulkes and Jefferson [16]. One hundred milligrams of cardiac left ventricle or skeletal muscle tissue was homogenized in a buffer containing 250 mM sucrose, 4 mM EDTA, 30 mM β-mercaptoethanol and 20 mM Tris–HCl, pH 8.3 in a homogenizer (Brinkmann PT3000, Mississauga, Canada) at half-maximal setting. The homogenates were then centrifuged at 12,000 × *g* for 15 min. The supernatants were subjected to sephadex G-50 gel filtration. It has been reported that the gel filtration step removes low molecular weight protein phosphatase inhibitors [17]. For this purpose, 500 μl of supernatant was loaded on a sephadex column and eluted with a buffer (50 mM Tris–HCl pH 7.7, 0.1 mM EDTA, 0.1% Brij 35 and 30 mM β-mercaptoethanol). The eluate obtained is defined as the tissue extract. All the above steps were carried out at 4 °C.

### 2.3. Measurement of phosphatase activities

Protein phosphatase activities were determined by using serine/threonine protein phosphatase assay kits from Upstate Biotechnology (Lake Placid, NY). The assay is based on the dephosphorylation of a synthetic phosphopeptide, KRpTIRR. The reaction was initiated by adding tissue extracts to the synthetic peptide substrate for 5 min and terminated by the addition of Malachite Green solution. The absorbance was read after 15 min at 660 nM to determine the inorganic phosphate released. The protein phosphatase activity measured here was completely inhibited by okadaic acid [18]. Since no agent is currently available to specifically inhibit PP1 or PP2A, it is difficult to accurately measure PP1 or PP2A activities.

### 2.4. Analysis of PP1 and PP2A protein contents

The relative content of PP1 and PP2A proteins in cardiac homogenates were determined by Western immunoblot analysis. Samples (20 μg) were separated on 12% gels by SDS-PAGE and electrophoretically transferred to Immobilon-P transfer membranes (Millipore, Bedford, MA). Membranes were probed with polyclonal antibodies recognizing PP1 (1:3000) and PP2A (1:2000) catalytic subunits (Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary antibodies were used and antigen–antibody complexes of all probed membranes were detected by the enhanced chemiluminesence detection system. The intensity of each band was scanned by an imaging densitometer with the aid of Molecular Analyst Software Version 1.3 (BioRad, Hercules, CA).

### 2.5. Data analysis

Values are mean ± S.E. and were statistically evaluated by one-way ANOVA. $P < 0.05$ was considered the threshold for statistical significance between groups.

## 3. Results

### 3.1. General characteristics and cardiac function

The data in Table 1 indicates that 8-week diabetic animals had significantly lower body weight and ventricular weight but exhibited a higher ventricular/body weight ratio in comparison to control animals. The induction of diabetes was confirmed by the presence of markedly elevated levels of plasma glucose and severely depressed levels of plasma insulin levels (in comparison to controls). Insulin treatment of the 4-week diabetic animals for a period of 4 weeks partially normalized the ventricular/body weight ratio and plasma glucose levels as well as completely normalized plasma insulin level. Cardiac dysfunction in diabetic animals was evident from significant depressions in the left ventricular +dP/dt and −dP/dt in comparison to controls

Table 1
General characteristics and cardiac function in control, diabetic and 8-week insulin-treated diabetic animals

| | Control | Diabetic | Diabetic + insulin |
|---|---|---|---|
| Body weight (g) | 542 ± 22 | 312 ± 21* | 354 ± 15 |
| Ventricular weight (g) | 998 ± 85 | 833 ± 40* | 882 ± 42 |
| Ventricular/body weight ratio (mg/g) | 1.84 ± 0.05 | 2.66 ± 0.01* | 2.49 ± 0.06# |
| Plasma glucose (mg/dl) | 165 ± 8.2 | 254 ± 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/s) | 5701 ± 244 | 3722 ± 183* | 4639 ± 266# |
| − dP/dt (mm Hg/s) | 5501 ± 320 | 3356 ± 118* | 4482 ± 253# |

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

(Table 1). These parameters were partially normalized after insulin treatment.

### 3.2. Cardiac muscle protein phosphatase activities

Table 2 indicates that 8-week diabetic animals had significantly higher cardiac muscle protein phosphatase activities in comparison to controls. However, the protein concentrations of cardiac muscle extracts from control and diabetic animals were not significantly different from each other. Insulin treatment partially normalized protein phosphatase activities in 8-week diabetic animals. The data in Table 2 also indicate that 8-week diabetic animals had significantly higher skeletal muscle protein phosphatase activities in comparison to controls. Insulin treatment completely normalized protein phosphatase activities in 8-week diabetic animals.

### 3.3. Relationship between changes in cardiac function and cardiac phosphatase activities

In order to examine the relationship between changes in cardiac function and cardiac protein phosphatase activities, a time course study was undertaken to measure these changes in 1-, 2-, 3- and 4-week diabetic animals. The data in Table 3 indicate significantly higher protein phosphatase activities in 1-, 2-, 3- and 4-week diabetic animals; the increase was maximal in 1-week diabetic animals. The results in Table 3 show that there was a progressive deterioration in cardiac function after the induction of diabetes. A significant decrease in − dP/dt was evident after 2 weeks of inducing diabetes, whereas + dP/dt was depressed after 3 weeks.

Table 2
Protein phosphatase activities in ventricular and skeletal muscle tissue extracts from control, 8-week diabetic and insulin-treated diabetic animals

| | Protein concentration (μg/mg heart) | Phosphatase activity (μmol Pi/min/mg protein) |
|---|---|---|
| *(A) Ventricular muscle* | | |
| Control | 56.7 ± 1.63 | 146 ± 5.5 |
| Diabetic | 57.0 ± 0.64 | 193 ± 5.0* |
| Insulin-treated diabetic | 56.2 ± 5.78 | 169 ± 7.6# |
| *(B) Skeletal muscle* | | |
| Control | 49.2 ± 1.63 | 50 ± 1.9 |
| Diabetic | 48.4 ± 0.64 | 69 ± 2.9* |
| Insulin-treated diabetic | 49.2 ± 3.78 | 43 ± 1.5# |

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

Table 3
Cardiac function and protein phosphatase activities of control, 1-, 2-, 3- and 4-week diabetic rat hearts

| | Cardiac function (mm Hg/s) | | Phosphatase activities (μmol Pi/min/mg protein) |
|---|---|---|---|
| | + dP/dt | − dP/dt | |
| Control | 6052 ± 217 | 5840 ± 243 | 146 ± 5.5 |
| 1 week | 5860 ± 248 | 5614 ± 208 | 256 ± 15.1* |
| 2 weeks | 5476 ± 225 | 4618 ± 174* | 203 ± 16.9* |
| 3 weeks | 4836 ± 180* | 4272 ± 158* | 201 ± 9.2* |
| 4 weeks | 4208 ± 164* | 3854 ± 142* | 199 ± 6.1* |

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

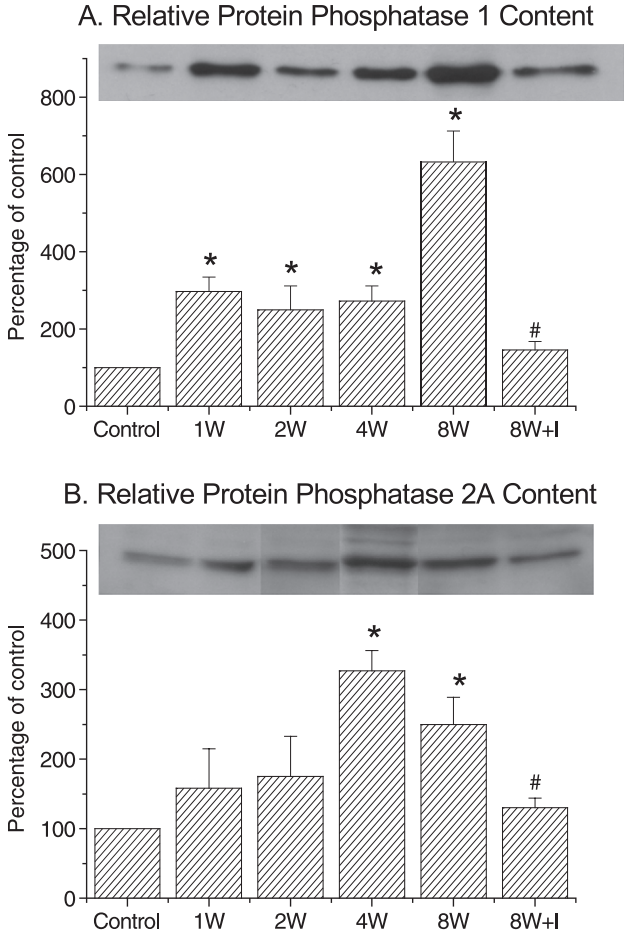


Fig. 1. Relative protein contents of PP1 (panel A) and PP2A (panel B) in control, 1-week (W), 2-week, 4-week, 8-week and insulin (I)-treated 8-week diabetic rat hearts. Each value is a mean ± S.E. of four experiments in each group. *$P<0.05$ vs. control; #$P<0.05$ vs. 8-week diabetic animals.

### 3.4. Relative protein content of cardiac PP1 and PP2A

Because cardiac protein phosphatase activities were increased in diabetic animals, the relative protein levels of PP1 and PP2A in cardiac muscle were examined in control animals, 1-, 2-, 4- and 8-week diabetic animals as well as insulin-treated diabetic animals. The typical protein bands are shown in Fig. 1. PP1 and PP2A protein bands were identified at 37 and 36 kDa, respectively. The densitometric analysis of bands of PP1 and PP2A revealed increases in the respective protein levels in 1-, 2-, 4- and 8-week diabetic animals in comparison to controls. Insulin treatment of 4-week diabetic animals significantly attenuated the elevation in PP1 and PP2A protein contents.

## 4. Discussion

The main finding of our study is that cardiac protein phosphatase activity was significantly elevated in streptozotocin-induced diabetic animals. An increase in protein phosphatase activity was also observed in the diabetic skeletal muscle. Our results are in contrast to two previous studies that reported no changes in cardiac protein phosphatase activities in diabetic animals [16,19]. The variance of results on cardiac phosphatase activities in diabetic animals between our study and an earlier study by Foulkes and Jefferson [16] could be due to the method of induction of diabetes—streptozotocin-induced diabetes (in our study) and alloxan-induced diabetes (in their study) [16]. This appears to be a distinct possibility because we followed the same tissue extraction method (gel filtration to remove the endogenous inhibitors) as described in the study by Foulkes and Jefferson in which no change in cardiac phosphatase activity was observed in 1-week diabetic animals. However, increased cardiac phosphatase activity observed in our current study is consistent with our earlier study [2] showing elevated cardiac cytosolic and SR-associated phosphatase activities in 6-week diabetic animals. It must be pointed out that gel filtration was not used in the latter study [2] and therefore the removal of low molecular weight inhibitors may not influence changes in phosphatase activities in the diabetic heart (in comparison to controls). With regards to disparity in results between our study and that of Jaspers and Miller [19], it must be pointed out that besides differences in the modes of induction in diabetes (alloxan vs. streptozotocin), Jaspers and Miller examined the activity of highly purified phosphatase 2A and not cardiac phosphatase activities in control and diabetic heart and therefore it is not possible to make direct comparisons. However, the lack of change in purified phosphatase 2A activity reported by Jaspers and Miller [19] after 1 week of diabetes is consistent with no change observed in protein content of cardiac phosphatase 2A in the diabetic heart after 1 week of diabetes in our study.

The elevation in cardiac protein phosphatase activities was seen as early as 1 week after the induction of diabetes, whereas abnormalities in cardiac function were evident after 2 weeks. The reduction in rate of cardiac relaxation ($-dP/dt$) observed after 2 weeks in our study is in contrast to a study by Tahiliani et al. [20], which used the same model (streptozotocin-induced diabetes) and reported that cardiac performance was impaired only after 6 weeks. The difference in results between both studies can be attributed to the technique of measuring heart function. In our study, the in vivo heart function was measured using the catheterization technique but Tahiliani et al. [20] assessed cardiac function ex vivo in isolated perfused hearts. Our results, however, are supported by studies that used the same model and assessed in vivo cardiac function by echocardiography or catheterization technique. A study by Mihm et al. [21], which characterized a detailed time course of changes in heart function using M-mode echocardiography reported diastolic dysfunction in diabetic rats as early as 3 days after streptozotocin administration. In another study Takeda et al. [22] showed a slight reduction (not statistically significant) in heart function (measured by the catheterization technique) after only 18 days of induction of diabetes (by streptozotocin administration) and significant decrease (in heart function) after 3 weeks. Thus, increased protein phosphatase activity preceding a depression in cardiac function suggests the possibility of enhanced protein dephosphorylation contributing to cardiac dysfunction in diabetic animals. Since the protein contents of cardiac PP1 and PP2A in diabetic animals were elevated, the observed increase in protein phosphatase activity might be due to increased expression of PP1 and PP2A. Because treatment of diabetic animals with insulin was found to prevent the increased protein phosphatase activity as well as PP1 and PP2A contents, it is evident that the observed changes in protein phosphatases are due to diabetes per se.

Major $Ca^{2+}$ handling and $Ca^{2+}$ regulatory proteins are known to be substrates for protein phosphatases. PP2A has been implicated in dephosphorylation of the PKA phosphorylation site on the L-type $Ca^{2+}$ channel that may result in decreased channel activity [23]. Thus, an up-regulation of PP2A may lead to marked reduction in the L-type $Ca^{2+}$ channel activity resulting in less $Ca^{2+}$, which is required for $Ca^{2+}$-induced $Ca^{2+}$ release from the SR in the diabetic heart. On the other hand, PP1 has been reported to dephosphorylate the PKA phosphorylation site on the RyR that may decrease SR $Ca^{2+}$ release channel activity [24]. Therefore, enhanced PP1 activity may cause a significant decrease in the SR $Ca^{2+}$ release channel activity resulting in less $Ca^{2+}$ available for myofibrillar activation in the diabetic heart. Both PP1 and PP2A have also been shown to dephosphorylate PLB at the PKA and CaMK phosphorylation sites that may result in reduced SR $Ca^{2+}$ uptake [15,25,26]. An increase in the levels of both protein phosphatases could thereby markedly depress SR $Ca^{2+}$ uptake and impair cardiac relaxation in the diabetic heart.

Okadaic acid, an inhibitor of PP1 and PP2A [17] has been reported to increase the phosphorylation state of TnI and thus, enhanced protein phosphatase activities could result in increased dephosphorylation of TnI and impairment of cardiac relaxation in the diabetic heart. The view is further substantiated by the observation that cardiac dysfunction and elevated levels of PP1 and PP2A in the diabetic heart were attenuated by insulin treatment. Thus, on the basis of these results, we conclude that an increase in PP1 and PP2A protein contents may contribute to cardiac dysfunction in diabetic animals. It must however be noted that phosphatase 2A might not be involved in the functional changes that begin early (after 2 weeks) but might contribute to changes taking place subsequently.

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