

## Full-length article

## Phosphorylated heat shock protein 27 is involved in enhanced heart tolerance to ischemia in short-term type 1 diabetic rats<sup>1</sup>

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### Key words

type 1 diabetes mellitus; ischemia; reperfusion; heart; heat shock proteins

<sup>1</sup> Project supported by Major State Basic Research Development Program of China (Grant No. G2000056905).

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Received 2005-02-03

Accepted 2005-02-24

doi: 10.1111/j.1745-7254.2005.00113.x

### Abstract

**Aim:** To examine the tolerance of type 1 diabetic hearts to ischemia and reperfusion injury. Myocardial contents of 27-kDa and 70-kDa heat shock proteins (hsp) as well as phosphorylated hsp27 were also determined. **Methods:** Hearts from hyperglycemic rats 3 weeks after streptozocin injection and age-matched normal rats were subjected to ischemia and reperfusion *in vitro*. Cardiac function and electrocardiogram were recorded throughout experiments. Myocardial heat shock proteins were detected with Western blot. **Results:** Despite depressed systolic function at the baseline, diabetic hearts exhibited considerable enhancement in post-ischemic heart function, manifested by an increase in the maximal rate of left ventricular pressure rise and fall (post-ischemic  $dp/dt_{max}$  and  $dp/dt_{min}$  were  $560 \pm 117$  and  $-313 \pm 68$  mmHg/s in control,  $n=7$ ,  $1249 \pm 57$  and  $-1204 \pm 36$  mmHg/s in diabetes,  $n=10$ ,  $P < 0.01$ ). Reperfusion ventricular fibrillation in the diabetic group were attenuated compared with controls ( $1.5 \pm 0.3$  vs  $7.2 \pm 2.1$  min in control,  $P < 0.01$ ). The increased heart resistance to ischemia in diabetes was associated with hyperglycemia and accompanied by enhanced expression of myocardial phosphorylated hsp27 with normal aortic vessel relaxation. Cardioprotection was abrogated by metabolic correction with insulin and accompanied by phospho-hsp27 reduction. **Conclusion:** Heart resistance to ischemia is increased in type 1 diabetes, and hyperglycemia may present a mild yet stressful stimulus leading to upregulation of endogenous stress protein, which may play a potential role in cardioprotection and compensate for detrimental effects of hyperglycemia in diabetes.

### Introduction

It is well known that cardiovascular disease is the major cause of morbidity and mortality in diabetic patients. However, there exists considerable debate over the causal relationship between diabetes *per se* and cardiovascular disease<sup>[1]</sup>. Unlike classical microvascular complications, large vessel atherosclerosis is not correlated with duration and severity of hyperglycemia<sup>[2,3]</sup>. Some studies demonstrate that cardiovascular disease prevalence remains constant despite different levels of glycohemoglobin. Furthermore, intensive control of hyperglycemia is not consistently effective

in reducing cardiovascular complications<sup>[4]</sup>, leaving unanswered the question of whether hyperglycemia contributes to cardiovascular disease in diabetes.

In addition to clinical debates, experimental studies also show inconsistent results regarding the role of hyperglycemia in myocardial ischemia. Studies of animals with type 1 diabetes demonstrate either increased or decreased sensitivity of diabetic hearts to ischemia<sup>[5-7]</sup>. Suggested mechanisms for decreased sensitivity include attenuated  $Na^+H^+$  exchanger activity, inhibited glycolysis, and reduced  $Ca^{2+}$  overload during reperfusion<sup>[7-9]</sup>. Studies demonstrating the detrimental effects of hyperglycemia on ischemia suggest

roles of pseudohypoxia and reactive oxygen species<sup>[6]</sup>. Regardless of whether hyperglycemia is the cause of increased cardiovascular disease, it is generally agreed that experimental type 2 diabetes has a detrimental effect on myocardial ischemia<sup>[10]</sup> and coexisting cardiovascular risk factors like hypertension and elevated cholesterol contribute significantly to the poor outcome in type 2 diabetes as seen in clinical settings<sup>[11,12]</sup>. Type 1 diabetes, however, has severe hyperglycemia and relatively less coexisting cardiovascular disease. Thus, type 1 diabetic animals provide good models for investigating hyperglycemia influence on ischemic outcomes.

The present study was designed to observe heart sensitivity to ischemia in type 1 diabetic rats using functional recovery and creatine phosphokinase as measures of ischemic injury, in addition to arrhythmias. Aortic vessel function was also examined to test whether there was functional impairment of large vessels. As circulating antibody titers to some of heat shock proteins family are increased in type 1 diabetic patients<sup>[13]</sup>, we observed whether these powerful protective proteins were upregulated in diabetic hearts. Our data indicate that type 1 diabetic hearts are resistant to ischemia/reperfusion with no obvious impairment of large vessel relaxation. The study also reveals increased expression of myocardial phosphorylated heat shock protein 27 (phospho-hsp27) in diabetes. To further examine the association of hyperglycemia and heat shock proteins with heart sensitivity to ischemia, we observed the effects of insulin treatment on ischemic outcomes and phospho-hsp27 by the correction of hyperglycemia.

## Materials and methods

**Animals** Male Sprague-Dawley rats (originally weighing 200–220 g) were injected intraperitoneally with 60 mg/kg of streptozocin (Sigma, St Louis, USA) in citrate buffer (pH 4.5). Age-matched control rats received an injection of citrate buffer. The onset of diabetes was confirmed by measuring blood glucose within 72 h after injection. Rats had free access to tap water and standard laboratory chow and were maintained until the *in vitro* experiment began after 3 weeks of hyperglycemia induction. Another experiment was carried out with one group of rats treated with subcutaneous injection of protamine zinc insulin (9 units/kg) 3 weeks after diabetes induction and a heart ischemia experiment was carried out after 2 d of complete correction of hyperglycemia (duration of insulin administration was  $5.1 \pm 0.7$  d). All experiments conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No 85–23, revised 1996).

**Isolated heart preparation and ischemia/reperfusion** The

hearts were excised under anesthesia with sodium pentobarbital 60 mg/kg, ip, and perfused retrogradely (Langendorff apparatus, IPH-W2, Labo Support, Osaka, Japan) through the aorta with Krebs-Henseleit buffer (KHB) containing: NaCl 118.0 mmol/L, KCl 4.7 mmol/L, CaCl<sub>2</sub> 2.5 mmol/L, MgSO<sub>4</sub> 1.2 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 1.2 mmol/L, NaHCO<sub>3</sub> 25.0 mmol/L, glucose 11.0 mmol/L, Na<sub>2</sub>-EDTA 0.5 mmol/L. After cannulation of the left atrium the hearts were switched to antegrade perfusion (working heart). Perfusate was kept at 37 °C and bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Aortic perfusion pressure was set at 60 mmHg and the left atrium at 10 mmHg at the beginning of each experiment. All hearts were allowed to beat spontaneously.

With a catheter inserted into the left ventricular cavity, left ventricular pressure (LVP), left ventricular end-diastolic pressure (LVEDP) and maximal rate of LVP rise ( $dp/dt_{max}$ ) and fall ( $dp/dt_{min}$ ) were recorded with a data acquisition system (MPA 2000, Alcott Biotech, Shanghai, China). The hearts were subjected to 30-min global ischemia by clamping both atrial inflow and aortic outflow and followed by 40 min of reperfusion.

Two silver electrodes were inserted into the myocardium and electrocardiogram was recorded continuously. Premature ventricular beats, ventricular tachycardia and fibrillation during reperfusion were recorded. The severity of arrhythmias was scored on a 0 to 9 scale<sup>[14]</sup>. The time from the cessation of perfusion to the cessation of heart contraction was recorded as time to arrest.

**Creatine phosphokinase and norepinephrine assay** Creatine phosphokinase (CPK) was measured in coronary effluent with assay kits (Jian-Cheng Biomedical Engineering, Nanjing, China) according to the manufacturer's protocols. Coronary norepinephrine was determined with high performance liquid chromatography coupled with electrochemical detection (Neurochem Model 5500, ESA, Bedford, MA, USA).

**Vessel tension recording** Aortic rings were suspended in an organ bath (PowerLab ML0140, ADInstruments, Castle Hill, NSW, Australia) containing KHB. The buffer was bubbled with 95% O<sub>2</sub> and 5% CO<sub>2</sub> and kept at 37 °C. Aortic rings were allowed to equilibrate for 90 min before tension recording. After precontraction with  $1 \times 10^{-7}$  mol/L of norepinephrine, acetylcholine (ACh) was added in a cumulative manner and aorta relaxation was recorded. Vasodilation in the presence of nitric oxide synthase inhibitor, *N*<sup>o</sup>-nitro-*L*-arginine methyl ester (*L*-NAME,  $1 \times 10^{-4}$  mol/L) was also measured.

**Western blot analysis** Left ventricles before and after ischemia were freeze-clamped and homogenized in lysis buffer (pH 7.4) containing: Tris/HCl 50 mmol/L, sucrose 150 mmol/L,

edetic acid-Na<sub>2</sub> 5 mmol/L, egtazic acid 2 mmol/L, Na<sub>3</sub>VO<sub>4</sub> 1 mmol/L, NaF 50 mmol/L, phenylmethanesulfonyl fluoride 0.1 mmol/L, leupeptin 1 mg/L. Protein samples were loaded onto 12% SDS-PAGE acrylamide gels and transferred to polyvinylidene difluoride membrane. Blots were incubated with antibodies to either hsp27 or phosphorylated hsp27 at serine 15 and inducible hsp70 (Santa Cruz, Santa Cruz, CA, USA). The immunoreactive bands were visualized using enhanced chemiluminescence detection, and relative levels of heat shock proteins were semiquantified with densitometry (QuantityOne software, Bio-Rad, Hercules, CA, USA) normalizing to actin band.

**Statistical analysis** Data were expressed as mean±SEM. Parameters were compared with analysis of variance (ANOVA) for repeated measures followed by Student-Newman-Keuls test. The relationship between blood glucose level and ischemic outcomes (arrhythmia score and time to arrest) was evaluated with multiple regression analysis.  $P<0.05$  was considered statistically significant.

## Results

**Effects of diabetes on baseline and post-ischemic heart function** A significant elevation in blood glucose was accompanied by a reduction in contractility of the diabetic heart, represented by lower  $dp/dt_{max}$  and lower heart work (lower left ventricular developed pressure, LVDP; the difference between LVP and LVEDP). Diabetic hearts also exhibited reduced heart rate and normal LVEDP at baseline perfusion (Table 1).

Although depressed at the baseline, post-ischemic re-

covery of  $dp/dt_{max}$ ,  $dp/dt_{min}$  and LVDP in diabetic hearts was enhanced significantly as compared with controls (Table 1). Coronary perfusion and cardiac output also recovered to better levels after ischemia in diabetes (Table 1). Reperfusion arrhythmias, cardiac CPK release, as well as cardiac norepinephrine release, were reduced in diabetic hearts (Table 2).

**Table 2.** Duration of arrhythmias, cardiac CPK and norepinephrine released from streptozocin-induced diabetic rat hearts underwent ischemia and reperfusion. Mean±SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control.

	Control ( <i>n</i> =7)	Diabetes ( <i>n</i> =10)
Duration of VT and VF/min	7.2±2.1	1.5±0.3 <sup>c</sup>
Coronary CPK/IU·g <sup>-1</sup> wet weight	642±73	349±90 <sup>c</sup>
Coronary norepinephrine/ pmol·g <sup>-1</sup> wet weight	258±44	121±37 <sup>b</sup>

VT and VF, ventricular tachycardia and fibrillation; CPK, creatine phosphokinase.

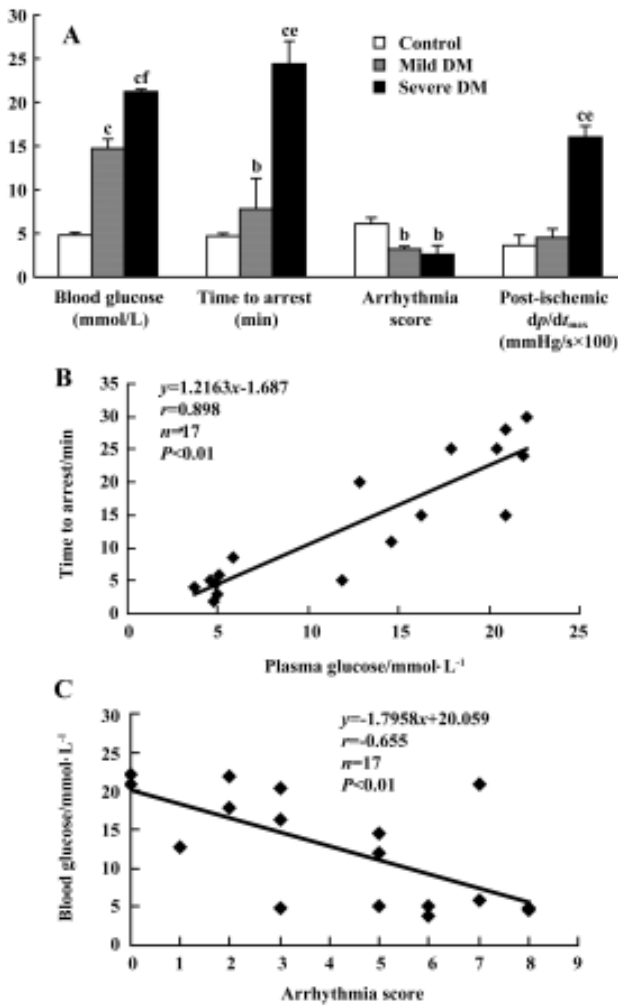
**Ischemic outcome and correlation with blood glucose level** Subgroups of mild or severe hyperglycemia (blood glucose <19 or >20 mmol/L) exhibited different post-ischemic outcomes (Figure 1A). Higher glucose within each group was accompanied by longer time to arrest and less arrhythmias (Figure 1B, 1C).

**Aortic relaxation** Normal vessel relaxation was found in the diabetic aorta (Figure 2), indicating no obvious impairment of large vessel function in this type of diabetic rat. The ACh-induced relaxation was abrogated by  $1\times 10^{-4}$  mol/L

**Table 1.** Characteristics, basal and post-ischemic heart function of streptozocin-induced diabetic rats and age-matched control rats. *n*=7 in control group. *n*=10 in diabetes group. Mean±SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs respective controls; <sup>e</sup> $P<0.05$  vs values in diabetes group before I/R.

	Before I/R		After I/R	
	Control	Diabetes	Control	Diabetes
Body weight (g)	306±9	198±14 <sup>c</sup>	–	–
Blood glucose (mmol/L)	4.8±0.3	18.0±1.6 <sup>c</sup>	–	–
Heart rate (beat/min)	219±15	163±23 <sup>c</sup>	121±23	97±14 <sup>e</sup>
Coronary flow (mL·min <sup>-1</sup> ·g <sup>-1</sup> )	14.4±0.7	13.6±1.1	6.2±0.6	8.1±0.5 <sup>e</sup>
Cardiac output (mL·min <sup>-1</sup> ·g <sup>-1</sup> )	41.5±2.3	38.5±3.8	9.1±2.3	15.3±1.6 <sup>e</sup>
LVDP (mmHg)	96±2	85±4 <sup>b</sup>	38.7±11.2	64.3±5.4 <sup>e</sup>
LVEDP (mmHg)	8.2±2.0	9.5±1.1	21.4±2.1	16.5±1.8 <sup>e</sup>
$dp/dt_{max}$ (mmHg/s)	2297±306	1603±117 <sup>b</sup>	560±117	1249±57 <sup>e</sup>
$dp/dt_{min}$ (mmHg/s)	-1491±178	-1418±108	-313±68	-1204±36 <sup>e</sup>

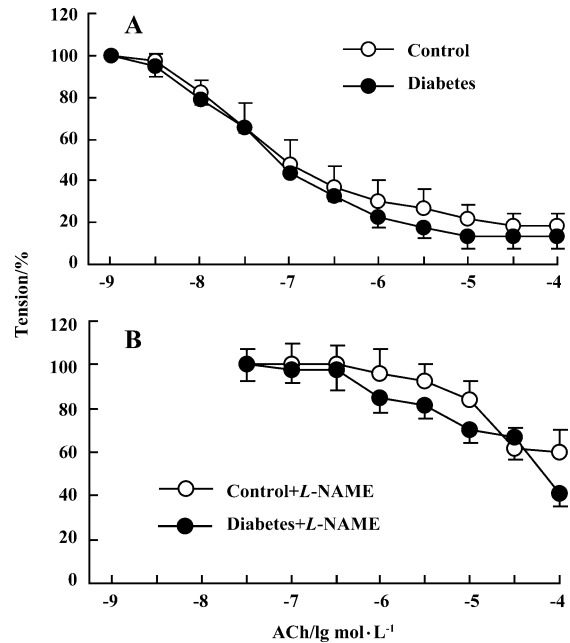
I/R, ischemia/reperfusion; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure.



**Figure 1.** Stratification of hyperglycemia and its relationship with ischemic outcomes. Higher blood glucose was associated with longer time to arrest, and higher post-ischemic  $dp/dt_{max}$  for diabetic rats (A,  $n=5-7$ . Mean $\pm$ SEM). Time to arrest was positively related to blood glucose levels *in vivo* (B) and arrhythmia score negatively related to glucose levels (C). Mild DM or severe DM, diabetic rats with blood glucose <19 or >20 mmol/L. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs age-matched controls; <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs age-matched mild DM.

*L*-NAME to similar extents in 2 groups.

**Myocardial expression of heat shock proteins** There were no differences in basal amounts of total hsp27 or inducible hsp70 between diabetic and control groups (Figure 3A, 3B). Because the protective ability of hsp27 depends on its phosphorylation state, we also analyzed phosphorylated hsp27 in the myocardium. Phospho-hsp27 content was found to be significantly higher in diabetic hearts before (Figure 3C) and after ischemia (Figure 3D). To eliminate the possible role of streptozocin in inducing expression of phospho-



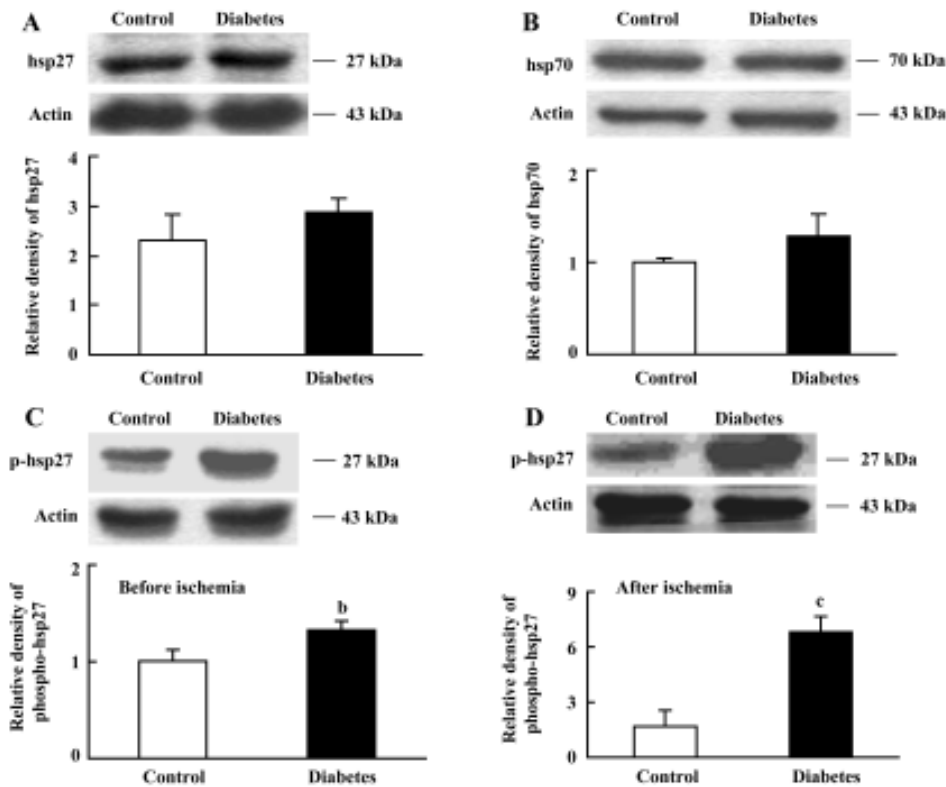
**Figure 2.** Aortic vasodilation in 3-week streptozocin-induced diabetic (DM) and age-matched control rats. There was no significant difference in aortic relaxation in response to acetylcholine (ACh) between diabetic and control rats (A). The vasodilation to ACh was partly abrogated by nitric oxide synthase inhibitor, *N*<sup>o</sup>-nitro-*L*-arginine methyl ester (*L*-NAME,  $1 \times 10^{-4}$  mol/L) to a similar extent in both groups (B).  $n=5$ . Mean $\pm$ SEM.

hsp27, we also examined the content of phospho-hsp27 in rats that failed to develop diabetes after streptozocin injection (15 % of animals receiving streptozocin did not show significant elevation of blood glucose in this study and served as non-diabetic controls). There was no significant change of phospho-hsp27 in those animals (relative density values for myocardial phospho-hsp27 before ischemia were  $5.4 \pm 0.9$  in normal controls vs  $6.2 \pm 1.0$  in non-diabetic controls receiving streptozocin,  $n=4$ ,  $P>0.05$ ).

**Ischemic outcomes after metabolic correction** Further intervention experiments were carried out with complete correction of hyperglycemia for 2 d prior to the ischemia experiment (Figure 4A). Cardioprotection was abrogated in blood glucose-controlled diabetes, demonstrated by increased reperfusion arrhythmias (Figure 4B) and reduced post-ischemic  $dp/dt_{max}$  (Figure 4C). Abrogation of cardioprotection through metabolic control was accompanied by reduction of myocardial phospho-hsp27 (Figure 4D).

**Discussion**

The present study demonstrated significantly enhanced resistance to ischemia/reperfusion in type 1 diabetic hearts,



**Figure 3.** Western blot analysis of heat shock proteins in left ventricles of 3-week diabetic rats. There was no significant difference in total hsp27 (A) and inducible hsp70 (B) between diabetic and control hearts before ischemia. Diabetic hearts showed higher levels of phospho-hsp27 before (C) and after (D) ischemia. Densitometric data are mean±SEM of 4 to 5 hearts. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs controls.

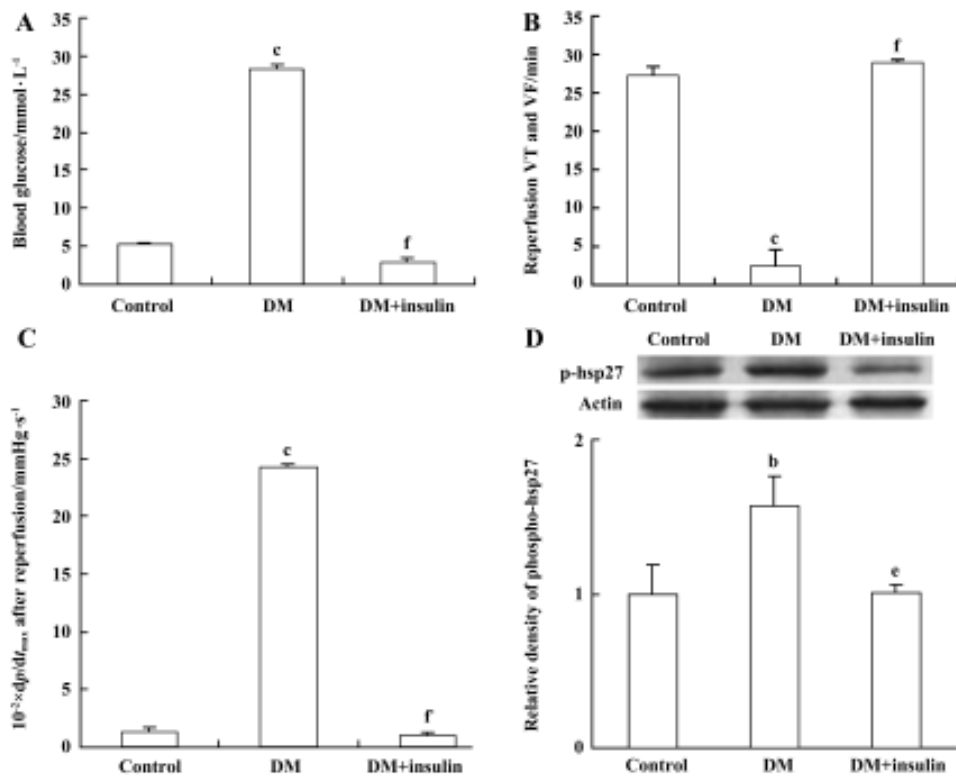
manifested by improved post-ischemic mechanical function, prolonged time to arrest, attenuated reperfusion arrhythmias and reduced CPK release. Furthermore, there was a significantly increased amount of phospho-hsp27 in diabetic hearts compared with non-diabetic hearts. The reduction of phospho-hsp27 in correlation to hyperglycemia was effective in abrogating heart tolerance to ischemia in diabetes.

Although there is controversy over the effects of hyperglycemia on cardiovascular outcomes, it is important to note that increased tolerance to ischemia is almost exclusively found in type 1 diabetic hearts<sup>[8,9,15,16]</sup>. In contrast to decreased sensitivity to ischemia of type 1 diabetic hearts, increased sensitivity to myocardial ischemia was found in type 2 diabetic Otsuka Long-Evans Tokushima Fatty rats<sup>[17]</sup>. It is of interest that higher blood glucose induced by sucrose feeding in type 2 diabetes paradoxically attenuated post-ischemic cardiac dysfunction<sup>[17]</sup>. There is also a study showing that the correction of metabolic state alone in type 2 diabetes did not improve heart function recovery from ischemia<sup>[10]</sup>. These data from type 1 and type 2 diabetes indicate that factors other than hyperglycemia increase heart sensitivity to ischemia in type 2 diabetes, and marked high

glucose or other factors secondary to elevated glucose can participate in preconditioning hearts to resist ischemia insult in type 1 diabetes.

A striking finding of the present study is that increased myocardial resistance to ischemia was accompanied by increased amounts of phospho-hsp27 in diabetic hearts. Because there is no significant change in myocardial phospho-hsp27 of rats that failed to develop diabetes after injection of streptozocin, the increase of phospho-hsp27 in streptozocin-induced diabetes is not likely to be induced by this toxic agent used for destroying pancreas beta-cells and induction of type 1 diabetes. Here we suggest for the first time that hyperglycemia can present a chronic and mild, yet stressful, stimulus to myocardium inducing upregulation of some stress proteins in diabetic hearts, which may in turn enhance heart tolerance to ischemia.

Elevated levels of hsp27 are reported to participate in cardioprotection by maintaining the integrity of microtubules and actin cytoskeleton, and can protect endothelium from ischemia<sup>[18]</sup>. The phosphorylation and translocation of hsp27 from cytosol to myofibril or nucleus is found to be especially important for protecting actin fragmentation and microtu-



**Figure 4.** Ischemic outcomes after insulin treatment. Complete correction of hyperglycemia with insulin (A) for 2 d abrogated protection in 3-week diabetic hearts subjected to ischemia as shown by increased arrhythmias (B) and reduced post-ischemic  $dp/dt_{max}$  (C) in insulin-treated diabetic rats (DM+Insulin). There was concomitant reduction of phospho-hsp27 as compared with diabetes control without insulin injection (DM) (D).  $n=5-6$ . Mean $\pm$ SEM. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs controls; <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs DM without insulin.

bule degradation<sup>[19]</sup>. In the present study, hyperglycemia did not affect total hsp27 but significantly increased the amount of phospho-hsp27, indicating the potential cardioprotective role of phospho-hsp27 during ischemia and reperfusion of diabetic hearts. The elevated lipid peroxidation product found in our previous work and other works<sup>[6,20]</sup> indicates higher oxidative stress in type 1 diabetes, while upregulation of protective proteins may compensate for such detrimental effects of hyperglycemia.

Inhibited glycolysis could result in myocardial salvage as a result of less accumulation of lactate during ischemia<sup>[21]</sup>. In accordance with previous published reports, we also found decreased lactate production from diabetic hearts<sup>[20]</sup>, which possibly contributed to the observed cardioprotection. In addition to the above mechanisms, decreased cardiac norepinephrine may also account for improved mechanical function, in accordance with our previous finding that norepinephrine reduction conferred significant cardioprotection during ischemia<sup>[22]</sup>. Because  $Na^+-H^+$  exchanger enhances norepinephrine release, diminished activation of  $Na^+-H^+$  exchanger in diabetic hearts<sup>[8]</sup> may be one explanation for

reduced norepinephrine release. This needs further investigation.

There is a report that early type 1 diabetes was found to have increased aortic relaxation<sup>[23]</sup>. In this study, no significant large vessel dysfunction was found in this early stage of type 1 diabetes. The relatively higher post-ischemic coronary flow in the diabetic group also indicated no impairment of coronary vessel function. The elevated coronary perfusion may be a result of myogenic dilation, a main determinant for myocardial perfusion, as during reperfusion the diabetic hearts relaxed significantly more than the controls. The improved coronary perfusion in turn enhanced myocardial salvage during reperfusion.

In summary, the present study reveals that the hearts of early-stage type 1 diabetes are resistant to ischemic injury. The concomitant upregulation of phospho-hsp27, in addition to inhibited glycolysis and reduced norepinephrine release, may participate in protecting diabetic hearts from ischemia. Hyperglycemia may contribute to the induction of the stress protein. However, caution should be taken when extrapolating the present results to clinical settings as most

diabetic patients have type 2 diabetes commonly associated with other risk factors. Further investigation on the combination of hyperglycemia and other conventional risk factors will lead to a better understanding of the complex pathogenesis of diabetes mellitus.

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