Type 2 diabetic obese *db/db* mice are refractory to myocardial ischaemic post-conditioning *in vivo:* potential role for Hsp20, F_1-ATPase δ and Echs1

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Received: March 31, 2011; Accepted: June 27, 2011

Abstract

Ischaemic post-conditioning (PostC) is a clinically relevant cardioprotective modality that has been confirmed in many species including human. It remains unknown if PostC can still protect heart in Type 2 diabetes, a rapidly growing disease in the world. This study investigated the efficacy of PostC in the leptin receptor–deficient *db/db* mice, which possess Type 2 diabetic characteristics including obesity, hyperglycaemia and hyperleptinaemia. Adult male C57BL/6J wild-type (*WT*) and *db/db* mice were anaesthetized, mechanically ventilated and subjected to left coronary artery occlusion for 30 min. followed by 24 hrs of reperfusion. For the PostC groups, the hearts underwent six cycles of 10 sec. of reperfusion and 10 sec. of re-occlusion at the onset of reperfusion. The mice were sacrificed at the end of 24 hrs reperfusion for infarct size measurement. PostC significantly reduced infarct size in *WT* mice ($n = 6$/group; $P < 0.05$), but not in the *db/db* mice. To identify alterations in protein expression by PostC, proteomic analyses were performed in the heart samples using two-dimensional differential in-gel electrophoresis with three CyDye labelling, followed by mass spectrometry. The results show that mitochondrial proteins (F_1-ATPase δ and Echs1) were down-regulated by PostC in *WT* heart. Such change was absent in the *db/db* heart. On the other hand, PostC reduced Hsp20 in the diabetic heart. In summary, PostC fails to protect Type 2 diabetic mice against ischaemia-reperfusion injury. The potential protein targets for the loss of PostC may include F_1-ATPase δ, Echs1 and Hsp20 that could regulate cellular ATP consumption/production and defense response to ischaemic stress.

Keywords: cardioprotection • ischaemia–reperfusion • myocardial infarction • proteomics • diabetes • hyperglycaemia • post-conditioning

Introduction

Since the first description of ischaemic PostC in an *in vivo* dog model of ischaemia–reperfusion (I–R) injury [1], this clinically relevant cardioprotective modality has drawn considerable interest and been confirmed in many mammalian species including human being [2] and mice [3–7]. The PostC has potential clinical applicability, it does not require the same pre-treatment timing restraint for ischaemic pre-conditioning and it could be used during the routine interventional reperfusion procedure in the patients with acute myocardial infarction. Recent studies in patients treated with primary percutaneous coronary intervention have further underscored such utility of PostC as a cardioprotective adjunct therapy for acute myocardial infarction [8, 9].

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doi:10.1111/j.1582-4934.2011.01376.x

Type 2 diabetes is a rapidly growing health problem affecting millions of the world population. This pathology constitutes a major independent risk factor for heart attack and many other cardiovascular diseases. However, only a limited number of studies have examined the effect of pre-conditioning in diseased hearts [10–12]. Similarly, vast majority of the studies on PostC have been performed in healthy animals and human beings. Another issue is that the exact cellular and molecular mechanism of PostC is still incompletely understood. In particular, very little information is currently available concerning the effect of PostC on cardiac protein expression under the Type 2 diabetic patients condition. To address this important issue, our current study was designed to investigate the efficacy of PostC in the membrane-bound leptin receptor deficient *db/db* mice, which possess many characteristics of Type 2 diabetes including obesity, hyperglycaemia and hyperleptinaemia. In addition, we undertook series of advanced proteomic studies, in which the two-dimensional differential in-gel electrophoresis with three CyDye labelling and mass spectrometry were used to explore novel protein targets that are altered by PostC and/or Type 2 diabetes. Our goal was to gain new insights to answer the fundamental question concerning the utility of PostC in protecting heart against I–R injury in Type 2 diabetic patients and the potential defects caused by Type 2 diabetes in cardiac protein response to PostC.

Materials and methods

Animals

Age-matched (10–12 weeks) adult male C57BL/6J wild-type mice and homozygous leptin receptor–deficient *db/db* mice (B6.BKS(D)-*Lepr*^{db}/J, stock number 000697) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, Revised 1996).

In vivo model of regional I–R injury and protocol for PostC

The mice were anaesthetized with pentobarbital sodium (70 mg/kg, i.p.), intubated and ventilated with a positive-pressure ventilator (MiniVent®Type 845; Harvard Apparatus, Holliston, MA, USA). The tidal volume was set at 0.2 ml, and the respiratory rate was adjusted to 133 cycles/min. All surgical procedures were carried out under sterile conditions. A left thoracotomy was performed at the fourth intercostal space, and the heart was exposed by stripping the pericardium. The left anterior descending coronary artery was then identified and occluded for 30 min. by a 7.0 silk suture placed around a small tube to obstruct blood flow. At the end of ischaemic period the tube was removed to allow reperfusion, followed by air expulsion and chest wall closure. The body temperature was maintained precisely at 37°C using a self-regulated mouse heating pad (catalogue no. NC9478922; Fisher Scientific, Pittsburg, PA, USA) throughout the entire surgical and post-operative periods. For the PostC groups, the hearts underwent six cycles of 10 sec. of reperfusion and 10 sec. of re-occlusion at the onset of reperfusion, a protocol of PostC that has been used in previous mouse studies [5, 7, 13].

Measurement of myocardial infarct size

After 24 hrs of reperfusion, the mice were re-anaesthetized and chest-opened to harvest the hearts, which were immediately cannulated *via* aortic opening and infused with saline for washing out the blood and then with 2 ml of 10% Evans blue dye for determining the area at risk and frozen under −20°C. Each of the hearts was sectioned into six to eight transverse slices (~1 mm thickness) and stained with 10% triphenyl tetrazolium chloride (TTC) for 30 min. at room temperature. The TTC-stained heart slices were then fixed with 10% formalin until measurement. The areas of infarct zone, the risk area and the entire left ventricle were determined by computer morphometric system with BIOQUANT® imaging software (BIOQUANT Image Analysis Corp, Nashville, TN, USA). Infarct size was expressed as percentage of the risk area.

Assessment of blood glucose level

Upon sacrificing each of the mice, a drop of blood sample was taken to measure individual blood glucose level using a Contour®blood glucose meter (Bayer HealthCare LLC, Mishawaka, IN, USA).

Proteomic analysis with 2D-DIGE and minimal CyDye labelling

In a parallel series of experiments, the heart samples were collected following the above-mentioned I–R protocol in the four experimental groups and immediately frozen under liquid nitrogen for proteomic analysis. The two-dimensional differential in-gel electrophoresis (2D-DIGE) and mass spectrometry protein identification were performed by Applied Biomics, Inc. (Hayward, CA, USA) following established protocols and the methodology, which were previously described in details [14].

Image scan and data analysis

Each gel was scanned immediately following SDS-PAGE using Typhoon Trio scanner (Amersham, Piscataway, NJ, USA). The scanned images were then analysed by Image QuantTL software (GE-Healthcare, Piscataway, NJ, USA), and then subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.5 (GE Healthcare). The change in ratio of differential protein expression was obtained from in-gel DeCyder software analysis. Quantitative comparisons were then made between two individual samples for each of the three possible combinations. In both *WT* and *db/db* heart samples, the pair-wise volume ratios (*i.e.* Control *versus* I–R, Control *versus* I–R + PostC, and I–R *versus* I–R + PostC) were calculated for each protein spot and used to determine relative protein expression. To simplify the presentation of the most relevant data, only the ratios of comparisons between I–R and I–R + PostC are presented for both *WT* and *db/db* hearts.

Spot picking and digestion and MALDI-TOF/TOF

The selected spots were picked up by Ettan Spot Picker (GE-Healthcare) following the DeCyder software analysis and spot picking design. The selected protein spots were subjected to in-gel trypsin digestion, peptides extraction, desalting and followed by MALDI-TOF/TOF to determine the protein identity. In brief, mass spectra (MS) of the peptides in each sample were obtained using Applied Biosystems Proteomics Analyzer. Ten to 20 of the most abundant peptides in each sample were further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. Protein identification was based on peptide fingerprint mass mapping (using MS spectra) and peptide fragmentation mapping (using MS/MS spectra). Combined MS and MS/MS spectra were submitted for database search using GPS Explorer software equipped with the MASCOT search engine to identify proteins from primary sequence databases.

Statistical analysis

The morphometric and physiological parameters (*i.e.* body weight, blood glucose and myocardial infarct size) are presented as mean ± S.E., which were analysed using one-way ANOVA followed by Student–Newman–Keuls *post-hoc* test for pair-wise comparison among the four experimental groups.

Results

Body weight, blood glucose and post-operative survival rate

The body weight was higher in the *db/db* mice as compared with the *WT* mice, with or without PostC (Fig. 1A; $P < 0.01$). Similar differences were also found in the blood glucose level ($P < 0.05$ *WT versus db/db*; Fig. 1B). The *in vivo* surgical protocol of I–R was performed in total of 20 *WT* mice and 42 *db/db* mice. The post-operative survival rates for each of the four experimental groups are: 72% *WT*, 77% *WT+PostC*, 35% *db/db* and 31% *db/db+PostC*. Approximately 70–80% of the mortality occurred between 10 min. and 1 hr after the beginning of reperfusion. The reperfusion-triggered severe ventricular arrhythmia and cardiac failure were the most likely causes of death. The mice surviving from the surgical procedure were randomly divided for either infarct size measurement or proteomic studies.

Myocardial infarct size

The area at risk (% of left ventricle, mean ± S.E.) was similar among all four groups, that is 43.2 ± 3.1 (*WT*), 41.2 ± 3.3 (*WT+PostC*), 41.7±1.3 (*db/db*) and 40.4±1.8 (*db/db+PostC*; $P > 0.05$). Myocardial infarct size was significantly higher in *db/db* group as compared with *WT* group ($P < 0.05$). PostC significantly reduced infarct size in the C57BL wild-type mice ($P < 0.05$, *WT versus WT-PostC*; Fig. 1C). In contrast, PostC failed to limit infarct size in the *db/db* mice (*db/db versus db/db-PostC*, $P > 0.05$). A set of representative TTC-stained transverse heart sections from each of the four experimental groups is shown in Figure 1C.

Modifications of protein expression in the normal *versus* Type 2 diabetic mice

Following 2D-DIGE, in-gel analysis was performed on the scanned images from each gel to determine the protein differential expression. Quantitative pair-wise comparisons of the protein volume ratios for each protein spot were made among the three treatment conditions (*i.e. Control versus I–R*, *Control versus I–R+PostC* and *I–R versus I–R+PostC*) in either *WT* or *db/db* mouse strains. Figure 2A shows an overlay image for in-gel comparison between the heart samples from the *WT* mice subjected to I–R (labelled in green dye) and to *I–R+PostC* (labelled in red dye). Similarly, Figure 1B depicts an overlay image for in-gel comparison between the heart samples from the *db/db* mice subjected to I–R (labelled in green dye) and *I–R+PostC* (labelled in red dye) groups. The numbered circles represent the protein spots that are differentiable among any pair of the treatment conditions in terms of abundance in protein expression using the 1.5 cut-off ratio.

As summarized in Table 1, four protein spots were significantly up-regulated (*i.e.* >1.5 fold) by PostC and six other protein spots were down-regulated by PostC in the *WT* hearts. Conversely, PostC in the *db/db* hearts led to increased expression in five proteins spots and reduction in 16 other protein spots. These spots were subsequently picked for protein identification using MALDI TOF/TOF tandem mass spectrometry.

Functional classification of the PostC-modified protein expression

To understand the functional significance of PostC-modified proteins in the heart, we grouped the positively identified proteins according to their known main biological function under the following six categories (Table 1). The first group is a mitochondrial antioxidant enzyme-peroxiredoxin 5, which was enhanced by PostC in both *WT* and *db/db* hearts. The second group composed of four cytoskeletal/contractile proteins, in which one protein—myosin regulatory light chain 2, cardiac isoform was up-regulated and two proteins (fibrinogen α and β) were down-regulated by PostC in both *WT* and *db/db* hearts. GAPDH isoform 2 was increased by PostC only in the *WT* heart. The third group included three proteins that play role in fatty acid or glucose metabolism. These proteins were modified by PostC only in the *db/db* heart (Table 1).

Most interestingly, in the fourth category, a low molecular weight stress protein—heat shock protein β-6 (HSPB6 or Hsp20) was significantly depressed for 2.32-fold by PostC only in the *db/db* hearts (Table 1). Furthermore, the fifth group of five

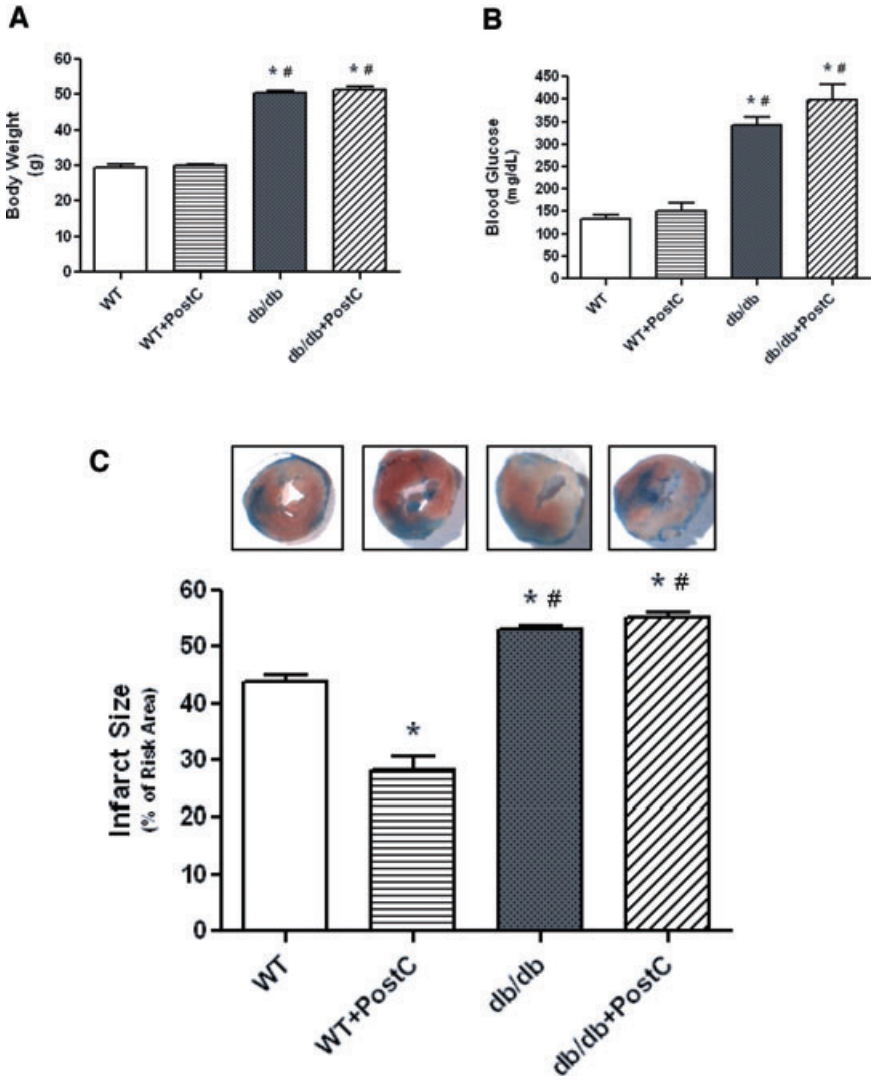

Fig. 1 Changes in body weight (**A**), blood glucose level (**B**) and myocardial infarct size (**C**) following *in vivo* regional ischaemia–reperfusion and ischaemic post-conditioning (*PostC*) in *WT* and Type 2 diabetic *db/db* mice. *WT*—C57/BL6J wild-type control group. $^{*}P < 0.05$ *versus WT* group; $^{\#}P < 0.05$ *versus WT+PostC* group.

mitochondrial proteins showed an opposing trend (*WT versus db/db*) in the protein abundance changes following PostC. In the *WT* group, two proteins (F1-ATPase δ and Echs1; highlighted with dashed line boxes in Table 1) were down-regulated by PostC and these changes were absent in the *db/db* group. On the other hand, three proteins related to mitochondrial respiratory chain were decreased by PostC in the *db/db* group, but not *WT* group (Table 1).

Finally, the sixth group consists of 11 proteins including 10 positively identified have less certain functional roles in the heart. Among these, seven proteins were down-regulated by PostC in the *db/db* hearts only. Most notably, a ~51 kD protein was enhanced by PostC for 3.44-folds in the *WT* heart only. The identity of this protein could not be determined.

Figure 3 shows representative DeCyder 3-D images highlighting the substantial differences in protein abundance between the I–R and *I–R+PostC WT* mouse hearts for F1-ATPase δ and Echs1.

Discussion

In this study, we show that PostC significantly reduced myocardial infarct size by 64% in the *WT* mice, but it was ineffective in protecting the Type 2 diabetic *db/db* mice against I–R injury. Although there has been mounting evidence for the cardioprotective efficacy of PostC in normal individuals of various species [1–7], there are only limited number of PostC studies in diseased animals. For example, it was reported that cardioprotective effect of PostC was blunted in aged mice [15], hypercholesterolemic rabbits [16] and leptin-deficient obese ob/ob mice [13]. This study (Fig. 1) also

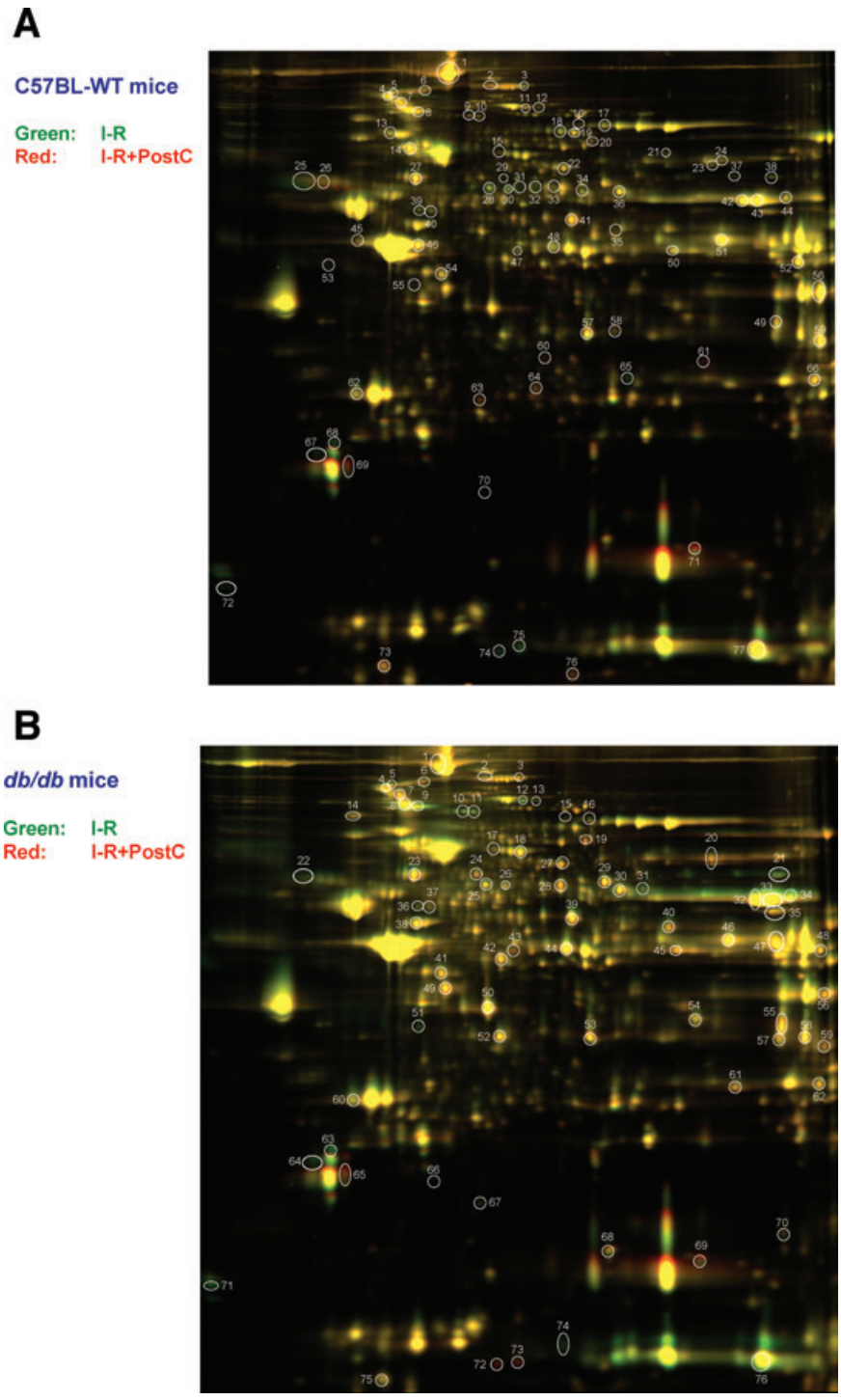

Fig. 2 Scanned images of the 2-D gels with three CyDye labelling. (**A**) Overlay image for in-gel comparison between the C57BL/6J wild-type mouse hearts from the ischaemia-reperfusion alone group (*I–R*; labelled in green dye) and the PostC-treated group (*I–R+PostC*; labelled in red dye). (**B**) Overlay image for in-gel comparison between the *db/db* diabetic mouse hearts from the *I–R* group (labelled in green dye) and the *I–R+PostC* group (labelled in red dye). The circled and numbered protein spots with a cut-off ratio of 1.5 for alteration in protein expression between the two treatment conditions are subsequently isolated from the gels and identified by MALDI TOF/TOF mass spectroscopy.

strongly suggests that the deficiency of long isoforms of leptin receptors (*db/db* mice) impedes the cardioprotective effects of PostC. Despite some common pathological features among the ob/ob and *db/db* mice, such as obesity and hyperglycaemia, there are also differences between these two mutant strains of mice. The most distinctive difference is that unlike the ob/ob mice that completely lack of leptin, the *db/db* mice actually have hyperleptinaemia [17]. Previous studies have shown increased mortality

Table 1 Ischaemic post-conditioning–induced modification of protein expression in the C57BL/6J wild-type and *db/db* diabetic mice

Protein name	Accession no.	MW (kD)	Wild-type (ratio to I/R)	*db/db* (ratio to I/R)
Antioxidant proteins				
Peroxiredoxin 5	gi|6746357	17.0	1.87 ↑	1.72 ↑
Cytoskeletal/contractile proteins				
Myosin regulatory light chain 2, cardiac isoform	gi|153791853	18.9	1.73 ↑	1.88 ↑
GAPDH isoform 2	gi|149251966	31.0	1.54 ↑	
Fibrinogen α polypeptide	gi|148683477	63.1	1.70 ↓	1.67 ↓
Fibrinogen β chain precursor	gi|33859809	54.7	1.71 ↓	1.56 ↓
Fatty acid/glucose metabolism proteins				
Pyruvate dehydrogenase [lipoamide]] kinase isozyme 4, mitochondrial precursor	gi|7305375	46.6		1.60 ↑
Long-chain-fatty-acid-CoA ligase 1	gi|729927	77.9		1.52 ↑
Apolipoprotein E precursor	gi|163644329	35.8		1.79 ↓
Stress proteins				
Heat shock protein β-6	gi|59958370	17.5		2.32 ↓
Mitochondrial proteins				
ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit	gi|148699643	14.3	2.65 ↓	
Echs1 protein	gi|12805413	31.2	2.19 ↓	
ATP synthase subunit α, mitochondrial precursor	gi|6680748	59.7		2.74 ↓
Ubiquinone biosynthesis protein coq7	gi|3806019	20.1		2.39 ↓
ATP synthase, H+ transporting, mitochondrial F1 complex, α subunit	gi|148677501	54.6		1.73 ↓
Other proteins with unknown function in heart				
Unnamed protein	gi|12843573	51.3	3.44 ↑	
Serum amyloid A (AA at 131)	gi|200915	10.3	3.05 ↓	
Serum amyloid A	gi|200904	12.9	2.18 ↓	
α-1-Globin	gi|553919	12.9		2.39 ↑
Plasminogen	gi|148670114	90.7		1.63 – 2.11 ↓
Gelsolin, isoform CRA_a	gi|148676699	82.5		1.78–1.98 ↓
Succinyl-CoA:3-ketoacid-coenzyme A transferase 1	gi|18266680	56.0		1.74 ↓
α-Actinin-2	gi|157951643	10.4		1.60 ↓
Protein S100-A9	gi|6677837	13.0		1.56 ↓
α-1-Globin	gi|553919	12.9		1.56 ↓
Serine (or cysteine) peptidase inhibitor	gi|15029662	45.9		1.53 ↓

Note: Data listed according to high-to-low fold change ratio of protein abundance between I/R+Postconditioning and I/R alone. The cut-off ratio of change was 1.50. MW: molecular weight; I/R: ischaemia/reperfusion.

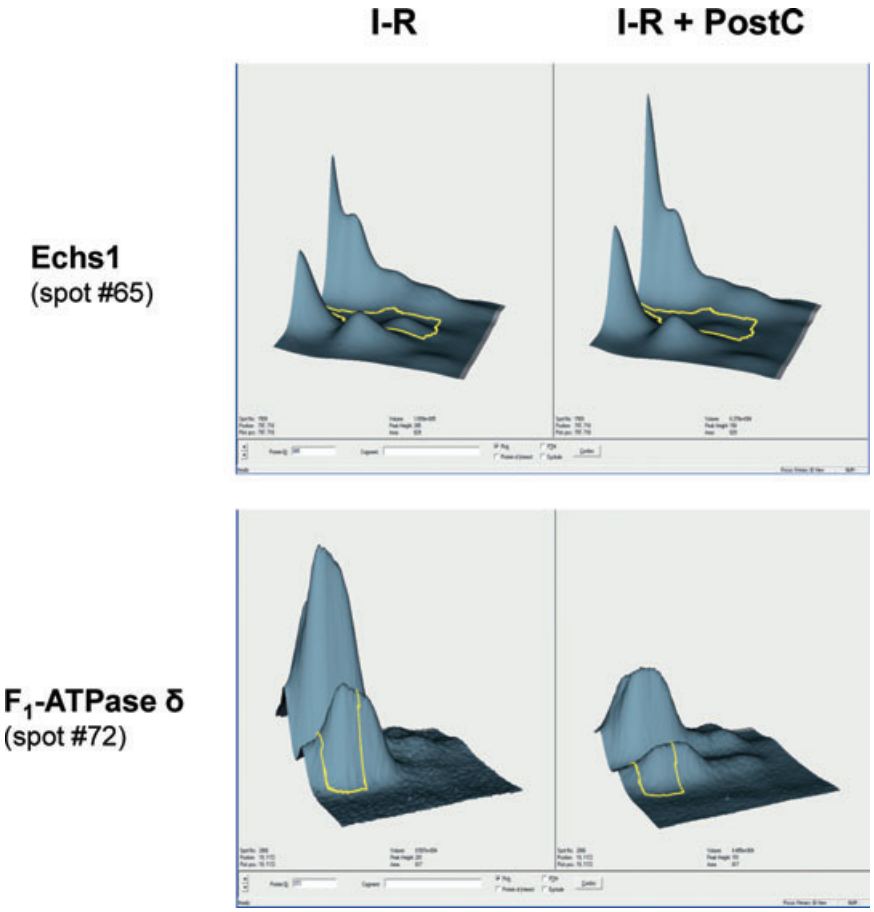

Fig. 3 Representative three-dimensional DeCyder software integrated graphs showing differences in protein abundance between the C57BL/6J wild-type mouse hearts subjected to ischaemia-reperfusion (*I–R*) alone (left) and to postconditioning intervention (*I–R+PostC*; right). Note that PostC remarkably down-regulated (F_1-ATPase δ) and Echs1 proteins.

[18] and acute renal failure [17] caused by endotoxin in the ob/ob mice, whereas the *db/db* mice were much more resistant to the detrimental effects of endotoxin [17]. Furthermore, hyperleptinaemia is a hallmark in human obesity [19] and the serum leptin levels are positively correlated with insulin resistance in Type 2 diabetic patients, after correction with age, gender and body fat mass [20]. Therefore, this study showing efficacy of PostC in *db/db* mice is important and more relevant to the human Type 2 diabetic patients' defects as compared with those in the ob/ob mice [13].

A recent study by Przyklenk *et al.* [21] also investigated PostC in both Type 1 and Type 2 diabetic mice. Although results of this study conceptually support our results in Type 2 diabetic mice (Fig. 1), there are several methodological differences worth mentioning. First, the strain of *db/db* mice was different, that is BKS.Cg-m+/+Lepr^{db}/J strain (The Jackson Laboratory stock number 000642) was used by Przyklenk *et al.* [21] *versus* B6.BKS(D)-*Lepr*^{db}/J strain (stock number 000697) used in this study. More importantly, we used an *in vivo* regional I–R model, in contrast to the *ex vivo* global I–R model in Langendorff isolated, buffer-perfused hearts in the other study [21]. Even though the *in vivo* model is a better mimic of the pathophysiological characteristics of the diabetic patients with acute myocardial ischaemia (*e.g.* the presence of hyperglycaemia and hyperleptinaemia during the course of regional myocardial I–R), the failure of PostC in the Type 2 diabetic *db/db* mice was identical regardless of the model differences.

Another major advance in this study is the proteomic identification of several novel protein targets that could be responsible the loss of PostC in Type 2 diabetic hearts. As shown in Table 1, the 17 kD heat shock protein β-6 (abbreviated as HSPB6 or most commonly Hsp20) was down-regulated 2.32-fold by PostC in the *db/db* heart only. Because the cardioprotective effect of this small heat shock protein has been demonstrated against I–R injury [22], we believe that the decreased Hsp20 expression in the db/db heart following PostC may be potential cause for the ineffectiveness of PostC in these mice. It is noteworthy that the levels of Hsp20 expression were similar in the *WT versus db/db* hearts under non-I–R baseline condition (data not shown). The two other proteins are δ subunit of mitochondrial F1 complex of ATP synthase (F_1-ATPase δ) and Echs1, which were down-regulated by PostC only in the *WT* heart. We speculate that the decreased F_1-ATPase δ would lead to reduced myocardial ATP consumption during reperfusion and the lower Echs1 would reduce β-oxidation of fatty

acids thereby limiting I–R injury [23]. Interestingly, under the non-ischaemic basal condition, Echs1 expression was not detectable in either *WT* or *db/db* hearts. However, F_1-ATPase δ was up-regulated in the *db/db* heart by 1.76-fold as compared with the *WT* control. Apparently, the higher level of F_1-ATPase δ in the Type-2 diabetic heart made the heart more vulnerable to I–R injury, as shown by the larger myocardial infarct size (Fig. 1C).

In addition, we observed four other proteins (*i.e.* peroxiredoxin 5, cardiac isoform of myosin regulatory light chain, fibrinogen α polypeptide and fibrinogen β chain precursor) that were modified by PostC in both *WT* and *db/db* hearts (Table 1). Although PostC-induced changes in these proteins were not sufficient to lead to infarct size reduction in the Type 2 diabetic hearts, their importance in PostC-induced protection should not be down-played. In particular, the mitochondrial antioxidant enzyme-peroxiredoxin 5 was found to play a role in nitrate-induced protection against doxorubicin cardiotoxicity [14].

Finally, it is noteworthy that our proteomic analysis was performed in the heart tissue samples collected at the end of 24 hrs reperfusion period, which is the same time-point for determining myocardial infarct size. Therefore, the differential responses in protein expression to PostC between the non-diabetic controls and Type 2 diabetic mice (Table 1) should reflect a steady-state changes in cardiac protein abundance rather than the transient changes in phosphorylation levels of several kinases (such as ERK, Akt and GSK3β) immediately following the PostC intervention. We did not intend to reconfirm the blunted early kinase response to PostC in diabetic hearts as previously shown [21], instead, we focused on identifying those stably modified protein targets throughout the prolonged (24 hrs) reperfusion period. It is well-known that the molecular determinants of myocardial infarct size are multi-factorial. There are injurious factors at the very onset of reperfusion, such as overproduction of reactive oxygen species (ROS), intracellular $Ca^{2\pm}$ overload and opening of mitochondrial permeability transition pore, which are known to be inhibited by PostC. However, it is much less clear how PostC influences myocyte viability during the later stage of prolonged reperfusion, in which the factors such as interstitial neutrophil infiltration and migration that can cause the secondary pro-inflammatory responses and persistent generation of ROS, which in turn contribute to the additional cardiac cell death and increase of infarct size. To our knowledge, this study is the first attempt to investigate the proteomic changes caused by PostC throughout the entire 24 hrs reperfusion period. Nevertheless, these proteomic findings alone do not confirm nor disapprove a causality for these differentially expression proteins in acquisition or loss of cardioprotection by PostC. It simply provides a non-biased clue for conducting further investigations on these positively identified protein targets.

In conclusion, this study provides first *in vivo* evidence that PostC fails to protect Type 2 diabetic *db/db* mice against I–R injury. These findings raise concerns of the potential utility of PostC in cardioprotection in Type 2 diabetic patients. Our study also identified three PostC-modified proteins (Hsp20, F_1-ATPase δ and Echs1) that were differentially regulated in the Type 2 diabetic animals as compared with the normal controls. Further studies are needed to elucidate the cause-and-effect relationship of these proteins and the loss of PostC in the diabetic hearts.

Acknowledgements

This study was supported in part by research grants from the National Institutes of Health (HL51045, HL79424, HL93685 to Dr. Rakesh C. Kukreja) and from the American Heart Association (National Scientist Development Grant No. 0530157N to Dr. Lei Xi). We thank Dr. John Liao of Applied Biomics, Inc., for his excellent technical support in proteomic analysis.

Conflict of interest

The authors confirm that there are no conflict of interest.

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