

Mitochondrial proteomics alterations in rat hearts following ischemia/reperfusion and diazoxide post-conditioning

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Abstract. Diazoxide post-conditioning (D-Post) has been shown to be effective in alleviating myocardial ischemia/reperfusion (I/R) injury; however, the specific mechanisms are not fully understood. In the present study, isolated rat hearts were subjected to I/R injury and D-Post. The mitochondria were extracted, and mitochondrial protein expression was detected in normal, I/R and D-Post hearts using two-dimensional electrophoresis and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Differentially expressed proteins were then identified using comparative proteomics. In total, five differentially expressed proteins were identified between the I/R and D-Post hearts. Compared with the I/R hearts, the expression of NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1), NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1), 2-oxoglutarate dehydrogenase (OGDH) and ATP synthase α subunit (isoform CRA_b, gil149029482) was increased in D-Post hearts. In addition, the expression of another isoform of ATP synthase α subunit (isoform CRA_c, gil149029480) was decreased in the D-Post group compared with the I/R group. The expression profiles of NDUFV1, NDUFS1 and OGDH in the two groups were further validated via western blotting. The five differentially expressed proteins may be protective effectors in D-Post,

as well as potential targets for the treatment of cardiac I/R injury.

Introduction

Ischemic heart diseases are amongst the leading causes of mortality worldwide (1-3), with a death rate of 46-324 deaths per 100,000 individuals/year (3). Early reperfusion is key for the effective recovery of the ischemic myocardium; however, reperfusion may also result in ischemia/reperfusion (I/R) injury (4). Under normal circumstances, mitochondria provide ATP for myocardial survival and contraction; however, they are also the site of myocardial oxidative stress and calcium overload during an I/R situation (4,5). Therefore, mitochondria are important factors (both preventative and causative) during myocardial I/R injury. Studies have shown that myocardial I/R injury and different types of conditioning can affect mitochondrial function (6-9). Furthermore, the expression levels of certain mitochondrial proteins are altered following myocardial I/R injury and ischemic preconditioning (10-12). Recently, it was reported that D-Post may protect Langendorff I/R hearts via the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) and the hypoxia-inducible factor-1/hypoxia response element pathway (13).

Following the discovery in 1991 of mitoK_{ATP} (14), which is located at the inner mitochondrial membrane, mitoK_{ATP} was demonstrated to be a trigger for the protective effects of ischemic pre-conditioning (15,16). Pharmacological conditioning is currently being evaluated as an alternative method for the treatment of I/R injury (17). Similar myocardial protection can be obtained from drugs such as mitoK_{ATP} openers, including diazoxide (15,18). A previous study observed that diazoxide post-conditioning (D-Post) is cardioprotective in I/R rat cardiomyocytes (12), which has been also reported by Penna *et al* (19). However, the mechanisms via which D-Post protects the myocardium against I/R injury have not been fully elucidated.

The aim of the present study was to analyze the differential expression of mitochondrial proteins in normal, I/R and D-Post rat hearts, which may aid the exploration of potential targets for the treatment of myocardial I/R injury.

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Materials and methods

Animals. A total of 65 male Sprague-Dawley rats (age, 16–20 weeks old, weight, 200–250 g) were purchased from the Center of Laboratory Animals of The Third Military Medical University (Chongqing, China). The rats were housed in cages with 12 h light/dark cycles, *ad libitum* access to food and water at a constant humidity (50–60%) and temperature ($22\pm1^{\circ}\text{C}$). All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (20), and all experimental protocols were approved by the Animal Care and Use Committee of Zunyi Medical University.

Materials. SDS, ammonium persulfate, sucrose, acrylamide, methylene bis-acrylamide, mannitol, glycerol and glycine were purchased from Amresco, LLC. Diazoxide, Nycodenz®, urea, thiourea, EDTA and tetramethylethylenediamine were obtained from Sigma-Aldrich (Merck KGaA). Protein quantification kits, immobilized pH gradient (IPG) strips, dithiothreitol (DTT), BIO-Lyte, CHAPS, agarose, bromophenol blue, β -mercaptoethanol, iodoacetamide and PVDF were acquired from Bio-Rad Laboratories, Inc. Anti-cytochrome *c* oxidase subunit IV (COX IV; cat. no. ab14744), anti-2-oxoglutarate dehydrogenase (OGDH; cat. no. ab137773), anti-NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1; cat. no. ab203208) and anti-NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1; cat. no. ab169540) antibodies were purchased from Abcam. All reagents were of analytical grade.

Perfusion protocol. Rats were intraperitoneally anesthetized using sodium pentobarbital (40 mg/kg) containing heparin (250 U/kg). When rats were in deep anesthesia and had lost the pinch reflex, rat hearts were rapidly excised and placed in cold K-H solution (2.50 mM CaCl_2 , 11.1 mM glucose, 4.75 mM KCl, 1.19 mM KH_2PO_4 , 118.00 mM NaCl, 1.19 mM $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ and 24.80 mM NaHCO_3 , pH 7.40). Then, hearts were quickly removed and connected to a Langendorff perfusion system. Hearts were perfused with 37°C K-H buffer bubbled for 10 min before perfusion with 5% CO_2 and 95% O_2 at 5.8 kPa for 20 min before equilibration. Exsanguination following heart removal resulted in rat mortality; death was confirmed from the loss of pinch reflexes and *rigor mortis* after the removal of heart. The control, I/R and D-Post hearts were perfused using the Langendorff apparatus as previously reported (9,21,22), and the protocols are outlined in Fig. 1. I/R injury was induced by hypoxia in the ischemia session and subsequent reperfusion with oxygenated K-H solution.

A total of 30 rat hearts were randomly allocated to the Control, I/R and D-Post groups ($n=10/\text{group}$). For equilibration, all hearts were perfused using the Langendorff apparatus with K-H solution for 20 min. Hearts in the Control group were continuously perfused with oxygenated K-H solution for 100 min. After equilibration, the I/R and D-Post hearts were subjected to 40 min of ischemia; the I/R hearts were then reperused with K-H solution for 60 min, while the D-Post hearts were reperused with diazoxide ($50\text{ }\mu\text{M}$ in K-H solution) for 2 min, and then with K-H solution for 58 min. Cardiac functional parameters, including heart rate (HR), the maximum rate of the rise in intraventricular pressure ($\text{dp}/\text{dt}_{\text{max}}$), left ventricular developed pressure (LVDP) and left

ventricular end-diastolic pressure (LVEDP) were recorded using the PowerLab system (ADInstruments) following equilibration (T1) and reperfusion (T2). At the end of equilibration, if premature systoles were $<2/\text{min}$, $\text{HR} >250\text{ bpm}$ and $\text{LVDP} >80\text{ mmHg}$, the ventricular tissues were collected for further experimentation at the end of the reperfusion period.

Transmission electron microscopy (TEM). The cardiac tissues were evaluated via electron microscopy, which was conducted as previously reported (9,23). Briefly, 1 mm^3 of the left ventricle was fixed in 0.25% glutaraldehyde and 3% paraformaldehyde at room temperature for 2 h. The tissues were then mounted with 1% osmic acid, dehydrated with acetone and embedded using ethoxyline 618 (35°C overnight, 45°C for 8 h, 60°C for 48 h). The myocardial sections were cut (50 nm thickness), stained in uranyl acetate and lead citrate for 30 and 10 min at room temperature, respectively, and then photographed with a transmission electron microscope (HITACHI-H7500; Hitachi, Ltd.) and ultrastructural damage was evaluated using Flameng's scoring method (24) as previously reported (9).

Mitochondria extraction. The purity of the extracted mitochondria is important for the accuracy of mitochondrial proteomics analysis. As previously described (9), the ventricular tissues were cut and placed into ice-cold mitochondrion-separating medium (700 mM sucrose, 210 mM mannitol, 1 mM EDTA and 10 mM Tris-HCl; pH 7.4), homogenized and centrifuged at $1,500\times g$ for 10 min at 4°C . The supernatant was then centrifuged at $12,000\times g$ for 10 min at 4°C to harvest the crude mitochondria. Finally, Nycodenz density gradient medium was layered into an ultracentrifuge tube (Beckman Coulter, Inc.) with layers of the following concentrations: i) 34% 0.5 ml; ii) 30% 0.8 ml; iii) 25% 1.2 ml (containing the crude mitochondrial solution); and iv) 20% 0.3 ml. The samples were then centrifuged at $100,000\times g$ for 60 min at 4°C to obtain purified mitochondria. To confirm the mitochondrial purity, TEM was performed to evaluate the status of the mitochondria.

Two-dimensional electrophoresis (2-DE) of mitochondrial proteins. To obtain the mitochondrial proteins, mitochondrial pellets were dissolved in hydration loading buffer (7 M urea, 2 M thiourea, 40 mM Tris base, 4% CHAPS and 1% DTT), sonicated for 10 sec, and centrifuged at $12,000\times g$ at room temperature for 20 min. 2-DE was performed as previously described (9,25) according to the manufacturer's protocol (Bio-Rad Laboratories, Inc.), but with minor modifications.

A 24-cm IPG strip (pH 5–8) was rehydrated for 14 h with hydration buffer containing $500\text{ }\mu\text{g}$ mitochondrial protein. Isoelectric focusing was carried out at 250 V for 1 h as follows: 1,000 V for 3 h, 4,000 V for 3 h, and then with incremental increases of 10,000 V until reaching 80,000 V/h. IPG strips were placed in 8 ml equilibration solution (375 mM Tris-HCl, 6 M urea, 20% glycerol, 2% SDS and 0.001% bromophenol blue) and protein separation was performed using a Bio-Rad system (Bio-Rad Laboratories, Inc.). The IPG strips were loaded onto a 12% SDS-PAGE gel in running buffer (192 mM glycine, 25 mM Tris and 0.1% mM SDS; pH 8.3), and a constant current was applied for 16 h.

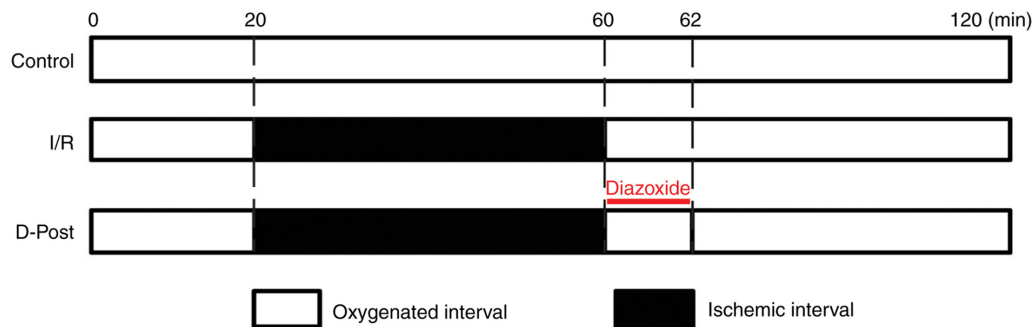


Figure 1. Perfusion protocol for cardiac I/R injury and D-Post. Hearts were categorized into three groups: i) Control; ii) I/R; and iii) D-Post. All hearts were equilibrated for 20 min before the different procedures were performed. Hearts in the Control group were continuously perfused for 100 min. I/R hearts were subjected to ischemia for 40 min and reperfused for 60 min. D-Post hearts received the same I/R treatment, and were then reperfused with K-H solution containing 50 μ M diazoxide for 2 min prior to reperfusion with K-H solution for 58 min. I/R, ischemia/reperfusion; D-Post, diazoxide post-conditioning.

Protein identification. Gels were stained with silver nitrate at room temperature for 30 min and digital images of protein dots on the gels were captured using a scanner (Seiko Epson Corporation). PDQuest 8.0 software (Bio-Rad Laboratories, Inc.) was used to identify differential spots between the Control, I/R and D-Post groups as previously described (9,25), and these differential protein spots were excised from the gels and digested. The peptide mass fingerprint was obtained using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) with a mass spectrometer (Ultraflex III; Bruker Corporation) and compared with that from the NCBI nr protein database (http://www.matrixscience.com/help/seq_db_setup_nr.html) as reported previously (9). Peptides were extracted with 50 mM NH_4HCO_3 :ACN (1:1, v/v). The peptide solution (3 μ l) was applied to a target disk to evaporate, and mixed with 0.1 μ l matrix solution (4 mg/ml in 70% ACN and 30% 0.1% TFA, v/v), spectra was obtained with MALDI TOF/TOF mass. BioTools 3.0 (Bruker Corporation) and Mascot software (Matrix Science, Inc.) were the databases used to identify proteins via peptide mass fingerprinting. NCBI nr was chosen as the sequence database. The names of the differentially expressed proteins were then confirmed.

Western blotting. Western blotting was conducted following standard procedures (26). The expression levels of β -actin, calnexin, GAPDH and COX IV in purified mitochondria were detected to confirm the purity of mitochondria used in the present study, and the expression levels of NDUFV1, NDUFS1 and OGDH were detected to compare with 2-DE results. Equal quantities of protein (60 μ g) from isolated mitochondria from Control, I/R and D-Post hearts were subjected to 12% SDS-PAGE, and transferred to PVDF membranes for immunoblotting. The membranes were blocked for 2 h at room temperature in TBS (20 mM Tris and 150 mM NaCl, pH 8.0), and then incubated at 4°C overnight with mouse anti- β -actin (cat. no. ab8226), rabbit anti-calnexin (cat. no. ab22595), mouse anti-GAPDH (cat. no. ab8245), mouse anti-COX IV (cat. no. ab33985), rabbit anti-NDUFV1 (cat. no. ab221998), rabbit anti-NDUFS1 (cat. no. ab169540) and rabbit anti-OGDH (cat. no. ab137773) primary antibodies (all 1:500 and purchased from Abcam). Then, membranes were subsequently incubated with HRP-conjugated anti-rabbit (cat. no. ab6721)

or anti-mouse (cat. no. ab6728) secondary antibodies (1:2,000; Abcam). Protein expression was visualized via enhanced chemiluminescence (Cytiva) with a ChemiDoc MP system (Bio-Rad Laboratories, Inc. Image Lab software (version 5.2.1; Bio-Rad Laboratories, Inc.), NDUFV1, NDUFS1 and OGDH levels were normalized to that of COX IV.

Statistical analysis. All data are expressed as the mean \pm standard deviation. Comparisons of protein expression among groups were conducted with one-way ANOVA followed by Sidak's post hoc test for multiple comparisons. Comparisons at different time points in the same group, and comparisons at the same time point in 3 groups were conducted with two-way mixed ANOVA followed by Sidak's post hoc test for multiple comparisons. Comparisons of Flameng's score among different groups were conducted using Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

D-Post improves cardiac function. D-Post effectively reversed I/R-induced hemodynamic dysfunction (Fig. 2). There was no significant difference in HR (Fig. 2A), $\text{dp/dt}_{\text{max}}$ (Fig. 2B), LVDP (Fig. 2C) or LVEDP (Fig. 2D) among the three groups at the end of T1. However, at the end of T2, the HR, $\text{dp/dt}_{\text{max}}$ and LVDP of the Control and D-Post groups were significantly greater than those of the I/R group, whereas the LVEDP was significantly decreased in these two groups compared with I/R ($P < 0.05$).

Electron microscopy. In the I/R group, the myocardial fibers were arranged in a disordered manner, the mitochondria were swollen, and the cristae were fractured and fuzzy (Fig. 3B). The myocardia of the D-Post group exhibited a more normal morphology; the myocardial fibers were arranged in an orderly manner, and fewer mitochondria were swollen (and to a lesser degree than those in the I/R group), but with an intact appearance (Fig. 3C). Quantification of mitochondrial damage was determined using Flameng's method (Fig. 3D). Flameng's score was considerably higher in the I/R group compared with the Control group (2.6 ± 0.46 vs. 1.3 ± 0.45), and subsequently decreased to 1.7 ± 0.48 in the D-Post group.

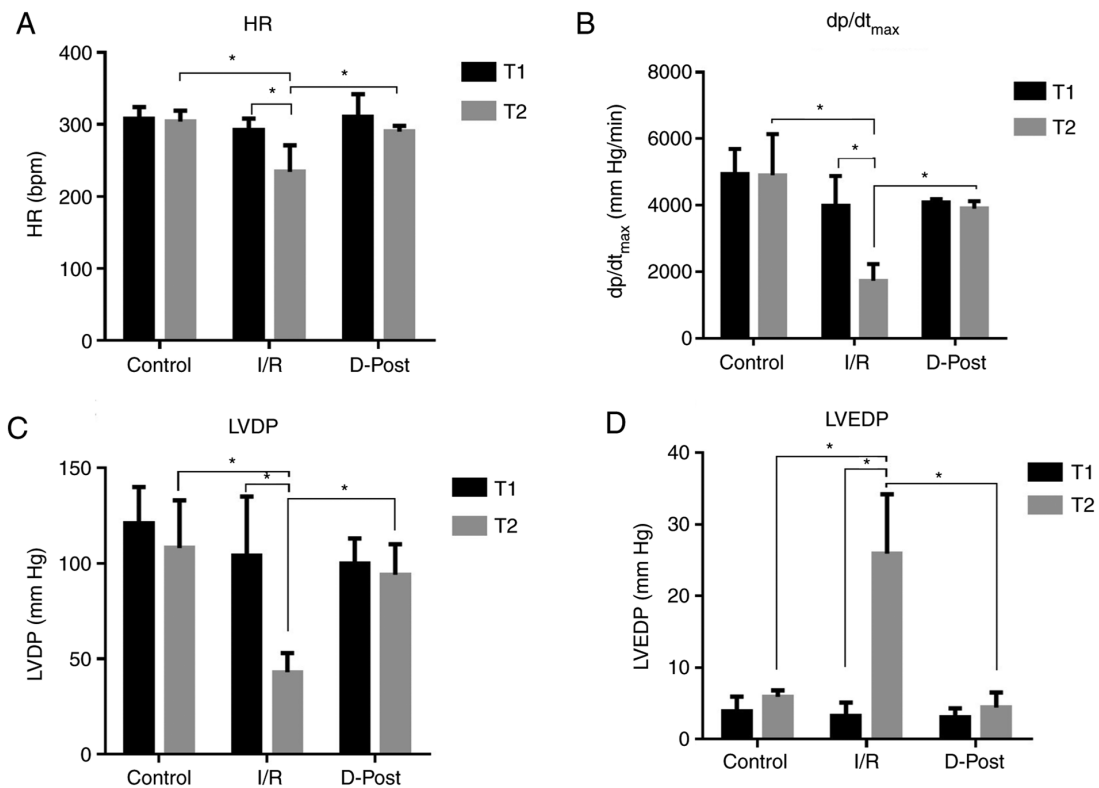


Figure 2. Comparison of hemodynamic parameters. D-Post effectively improves (A) HR, (B) dp/dt_{max} , (C) LVDP and (D) LVEDP after I/R injury. $n=10$ in each group. Comparisons at different time points in the same group, and comparisons at the same time point in different groups were conducted using two-way ANOVA followed Sidak's post hoc test. * $P<0.05$. I/R, ischemia/reperfusion; D-Post, diazoxide post-conditioning; HR, heart rate; dp/dt_{max} , maximum rate of the rise in intraventricular pressure; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; T1, time point following equilibration; T2, time point following reperfusion.

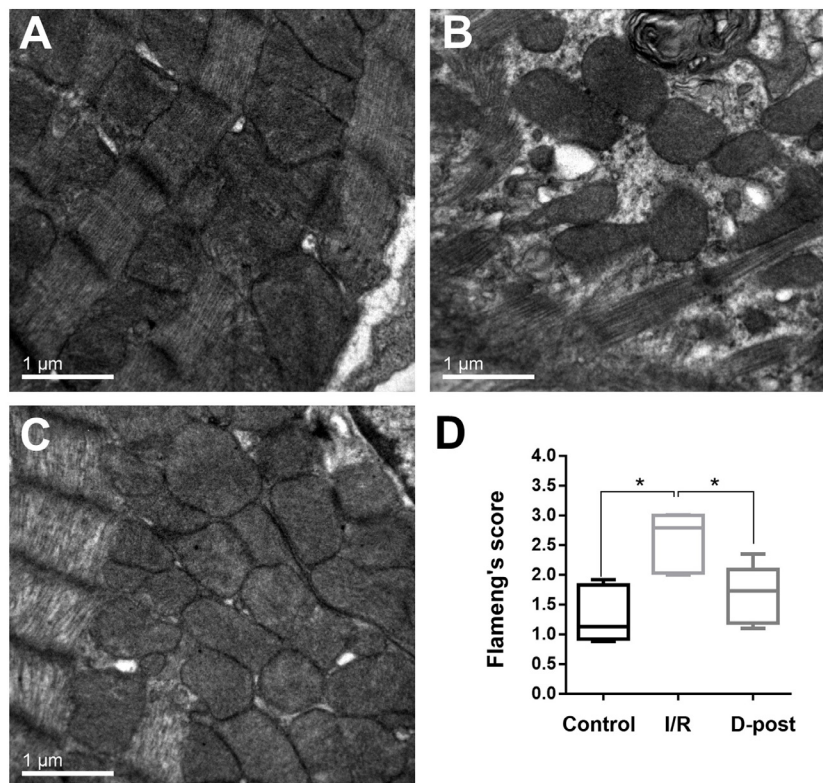


Figure 3. TEM analysis of myocardial damage. TEM images of Langendorff-perfused myocardia were analyzed following reperfusion in the (A) Control, (B) I/R and (C) D-Post groups. (D) Flameng's score of mitochondria from TEM images ($n=4$ /group). Comparisons among different groups were conducted using Kruskal-Wallis test followed by the Dunn's post hoc test. * $P<0.05$. TEM, transmission electron microscopy; I/R, ischemia/reperfusion; D-Post, diazoxide post-conditioning.

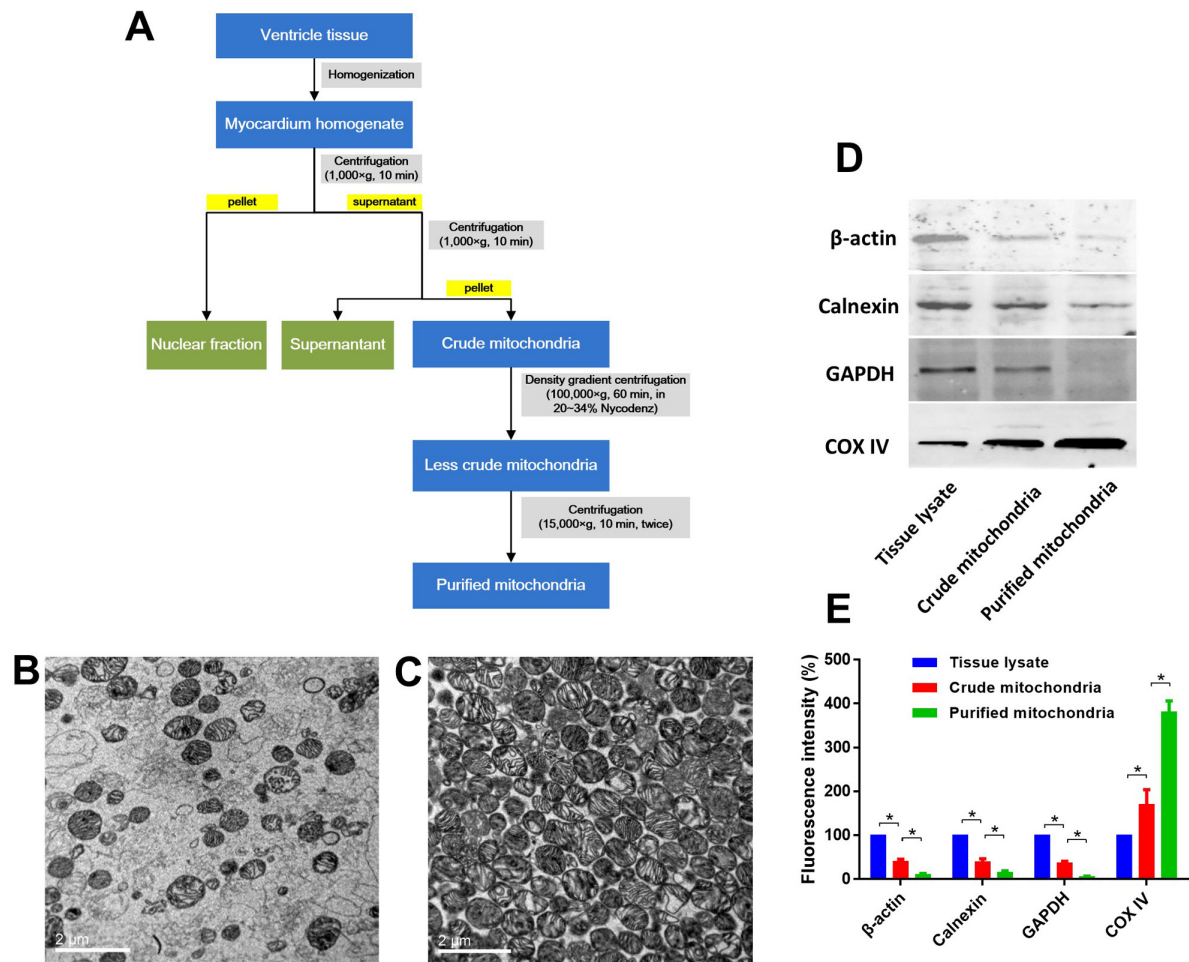


Figure 4. Flow chart of mitochondrial extraction and purification protocols. (A) Mitochondria were isolated using the Nycodenz density gradient centrifugation method. Representative transmission electron micrographs of (B) crude and (C) purified mitochondrial fractions from a heart in I/R group. Scale bar, 2 μ m. (D) Evaluation of the expression of β -actin, calnexin, GAPDH and COX IV in the purified mitochondria via western blotting. (E) Expression levels of β -actin, calnexin and GAPDH in purified mitochondria were significantly decreased compared with in heart tissue and crude mitochondria, while the expression levels of COX IV in purified mitochondria were increased. $n=4$ in each group. $P<0.05$. I/R, ischemia/reperfusion; D-Post, diazoxide post-conditioning; COX IV, cytochrome *c* oxidase subunit IV.

Evaluation of mitochondrial purity. Mitochondria were extracted and purified as depicted in Fig. 4A. Mitochondrial purity was evaluated using TEM images and western blotting of β -actin, calnexin, GAPDH and COX IV, which indicated that high purity mitochondria were obtained (Fig. 4B-E). Representative TEM micrographs of the isolated mitochondria are displayed in Fig. 4. The mitochondria were intact with no swelling or rupturing, and the cristae were intact and organized (Fig. 4C). Few impurities were present within these fields of view (Fig. 4C). Western blotting data also indicated that the purified mitochondria exhibited high purity (high expression of COX IV, and low expression of β -actin, calnexin and GAPDH), as shown in Fig. 4D and E.

D-Post alters mitochondrial protein expression. The expression levels of mitochondrial proteins in the I/R and D-Post groups were compared following 2-DE. The gels were stained with silver nitrate and scanned, and representative images are presented in Fig. 5A (I/R) and 5B (D-Post). In total, 14 spots were identified with expression differences $>50\%$ between the two groups (Fig. 5C).

Protein identification with MALDI-TOF MS. A total of 14 spots were isolated from the 2-DE gels of the I/R group, and subjected to MALDI-TOF MS. The peptide mass peaks were compared with those in the NCBI nr protein database, revealing five differentially expressed proteins [NDUFV1, NDUF51, OGDH, ATP synthase (isoform CRA_c, isoform CRA_b)]; descriptions of these five proteins are listed in Table I.

PPC alters the mitochondrial expression levels of NDUFV1, NDUF51 and OGDH. To validate the data obtained from 2-DE and MALDI-TOF MS, three of the five differentially expressed proteins were selected and subjected to western blotting. The expression levels of NDUFV1, NDUF51 and OGDH in the D-Post group were significantly upregulated compared with in the I/R group ($P<0.05$, Fig. 6), consistent with the 2-DE expression trends for these proteins.

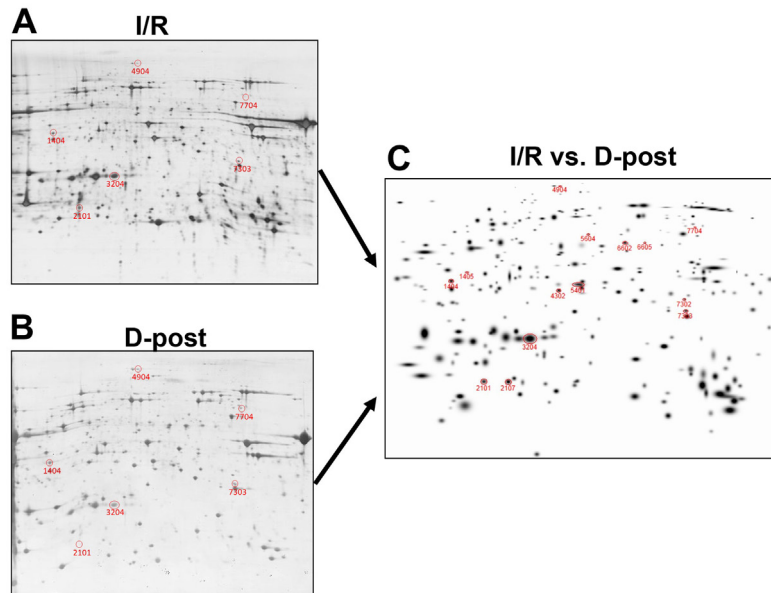
Discussion

D-Post was previously revealed to effectively attenuate I/R injury in primary adult rat cardiomyocytes (12). In the present *ex vivo* study, D-Post alleviated I/R injury in rat hearts.

Table I. Information of differentially expressed proteins.

Protein name	Gene name	Calculated PI	SSP ^a	NCBI GI no.	AA coverage ^b , %	Fold-change ^c	P-value ^d
NADH dehydrogenase (ubiquinone) flavoprotein 1	NDUFV1	5.89	1405	149061921	24	3.6±0.2	0.002
2-oxoglutarate dehydrogenase	OGDH	6.30	4904	62945278	12	3.3±0.5	0.005
NADH-ubiquinone oxidoreductase 75 kDa subunit	NDUFS1	5.74	5604	149046009	24	2.6±0.5	0.008
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, α subunit, isoform 1, isoform CRA_c	ATP5F1A	9.50	7704	149029482	25	2.5±0.8	0.003
ATP synthase, H ⁺ transporting, mitochondrial F1 complex, α subunit, isoform 1, isoform CRA_b	ATP5F1A	6.92	2107	149029480	38	-2.4±0.7	0.001

^aProtein spot number provided by PDQuest; ^bAA coverage rate of proteins compared with reference proteins in the NCBI protein database; ^cFold-change in D-Post group compared with I/R, n=4; negative values indicate downregulation in D-Post group; ^dT-test of expression (in parts per million) between I/R and D-Post groups. D-Post, diazoxide post-conditioning; GI, GenInfo Identifier; I/R, ischemia/reperfusion; NCBI, National Center for Biotechnology Information; PI, isoelectric point; SSP, significant spot.



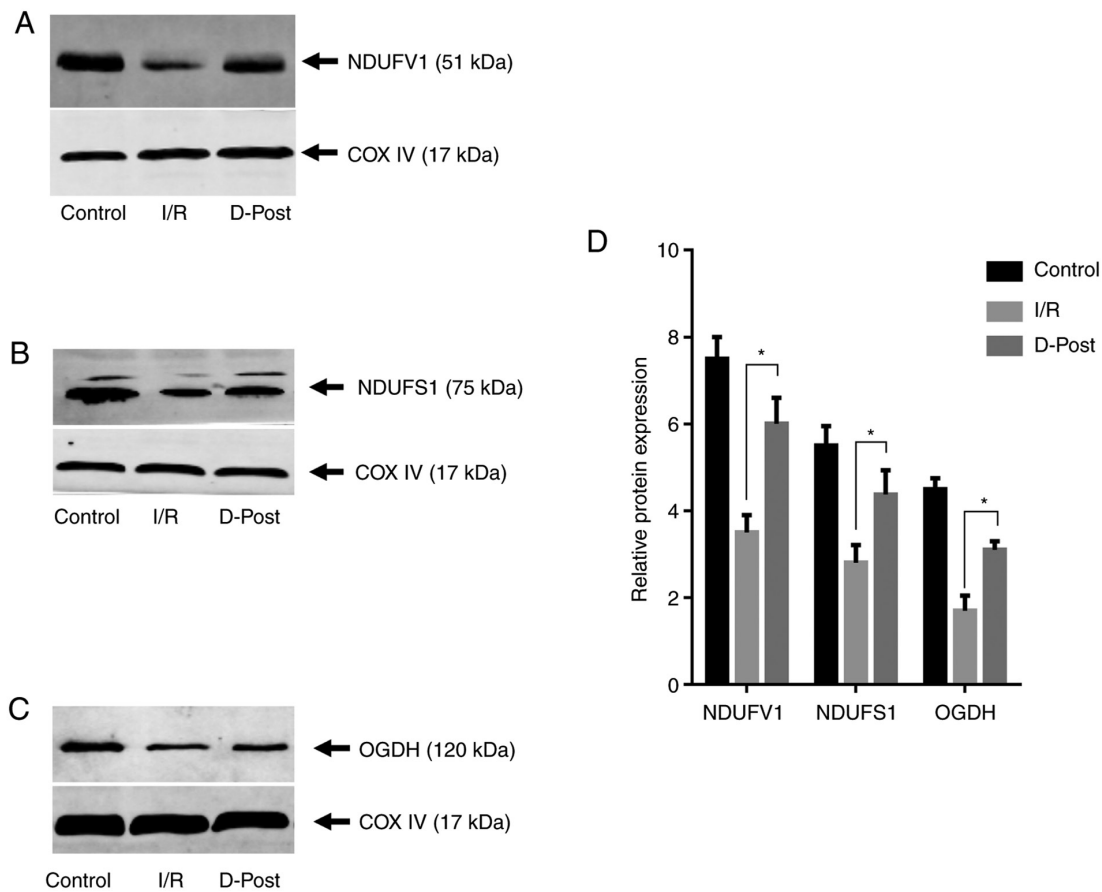


Figure 6. Expression levels of NDUFV1, NDUFS1 and OGDH in normal, I/R and D-Post hearts. (A) NDUFV1, (B) NDUFS1 and (C) OGDH expression as determined via western blotting. (D) Compared with the I/R group, expression of each protein was upregulated in the D-Post group, which was consistent with the expression trends observed with two-dimensional electrophoresis. $n=3$ in each group. * $P<0.05$. I/R, ischemia/reperfusion; D-Post, diazoxide post-conditioning; COX IV, cytochrome *c* oxidase subunit IV; NDUFV1, NADH dehydrogenase (ubiquinone) flavoprotein 1; NDUFS1, NADH-ubiquinone oxidoreductase 75 kDa subunit; OGDH, 2-oxoglutarate dehydrogenase.

decarboxylation of α -ketoglutarate to succinyl-CoA, NADH and CO_2 (32). In addition, OGDH also regulates mitochondrial redox potential (NADH/NAD $^+$), and has been reported to be a significant source of reactive oxygen species (ROS) during mitochondrial succinate metabolism in the porcine heart (33). OGDH expression is reportedly decreased in the I/R heart (10,34), which may influence ROS and energy production in the myocardium. In the present study, 2-DE and western blotting revealed an increase in OGDH expression following D-Post, and this recovery in expression may restore ROS and energy homeostasis in the I/R heart, thus decreasing damage to the ischemic myocardium.

The expression of ATP synthase subunit α (ATPA) was found to be altered in I/R hearts subjected to D-Post. Of note, two isoforms of ATPA were revealed to be differentially expressed; the CRA_c isoform was upregulated in D-Post hearts, while the CRA_b isoform was downregulated. As there are no specific antibodies that differentiate between these two isoforms, their protein expression levels cannot be separately detected by western blotting. I/R has been reported to promote the inhibition of ATP synthase, and subsequently decrease ATP production (35,36). In addition, ATP synthase contributes to the effects of mitoK $_{\text{ATP}}$ opening in I/R hearts (37,38). D-Post increases ATP synthase activity, which may be a direct reason for its cardioprotective effects (38). However, whether

the effects of D-Post are specifically associated with ATPA is yet to be investigated.

The present proteomics analysis of mitochondrial proteins between I/R and D-Post rat hearts is a practical way to highlight potential D-Post effector proteins. A total of five differentially expressed proteins were identified using 2-DE and MALDI-TOF MS (and validated via western blotting), and each warrants further investigation. Proteomic changes between normal Langendorff and I/R hearts have been investigated previously and 4 differentially expressed proteins (ATPA, isoform 2 of cytochrome *c*1, electron-transferring flavoprotein and NDUFS2) have been identified with the same protocol (25). Therefore, the differentially expressed proteins between the Control and I/R groups were not investigated in the present study.

There were limitations to the present study: i) The function of these differentially expressed proteins in I/R and D-Post tissues were not studied further (for example, it is not clear whether interventions in the expression of these proteins may affect I/R injury in the myocardium); ii) the use of narrow-range pH IPG strips (pH 5-8) may result in other potential proteins not being detected, which may be the reason that only five differentially expressed proteins were identified between the I/R and D-Post hearts; iii) for the evaluation of the protective effects of D-Post, triphenyltetrazolium chloride staining of

Langendorff hearts would be a more convincing indicator and should be used to measure myocardial infarct area; and iv) as mentioned above, the lack of a normal control group for the 2-DE experiments is a limitation of the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YP and YW confirm the authenticity of all the raw data. YP, YW, WS and SC performed the experiments, wrote the manuscript and prepared the figures. SC, YL and TY conceived the study, and provided the reagents and materials. All authors reviewed the data and drafts of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals in China, and all experimental protocols were approved by the Animal Care and Use Committee of Zunyi Medical University (Zunyi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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