Int J Clin Exp Med 2019;12(5):5060-5068
www.ijcem.com /ISSN:1940-5901/IJCEM0087455

Original Article

Potential biomarkers for myocardial ischemia-reperfusion injury and pinacidil post-conditioning identified with mitochondrial proteomics in rats

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Received October 27, 2018; Accepted February 12, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Pinacidil post-conditioning (PPC) has been shown to be effective in alleviating myocardial ischemia-reperfusion (I/R) injury. However, the underlying molecular mechanisms are not fully elucidated. Langendorff-perfused isolated rat hearts were subjected to global I/R and received PPC. Myocardial mitochondria was extracted and mitochondrial protein expression was detected in normal, I/R, and PPC rat hearts using comparative proteomics, i.e. two-dimensional electrophoresis (2-DE) and MALDI-TOF MS. Four differentially-expressed proteins were identified between normal and I/R hearts, as well as 7 between PPC hearts and I/R hearts. These proteins were identified as ATP synthase subunit alpha (ATPA), isoform 2 of cytochrome c1, electron transfer flavoprotein subunit alpha (ETFA), NADH dehydrogenase [ubiquinone], iron-sulfur protein 2 (NDUFS2), elta(3,5)-delta(2,4)-dienoyl-CoA isomerase (ECH1), ATP synthase subunit d (ATPD), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (NDUFA10), isocitrate dehydrogenase [NAD] subunit alpha (IDHA), and NADH dehydrogenase [ubiquinone] flavoprotein 2 (NDUFV2). Interestingly, two interested protein spots in the 2-DE gels were identified as ATPD between the PPC group and I/R group. These differential proteins could be potential biomarkers for hearts undergoing I/R injury and receiving PPC.

Keywords: Pinacidil post-conditioning, myocardial ischemia/reperfusion injury, mitochondria, proteomics

Introduction

Ischemic heart disease remains the leading cause of deaths, worldwide [1-3]. Early reperfusion is the key to treatment of this disease, but inevitably leads to myocardial I/R injury. Therefore, understanding the molecular mechanisms of I/R injury and discovering methods to attenuate this disease are particularly important. Pinacidil is a nonselective and mitochondria-sensitive potassium channel (mito-KATP) opener. Previous studies have shown that PPC can reduce myocardial I/R injury [4-6]. However, mechanisms by which pinacidil conditioning (PPC) protects the myocardium against I/R-induced injury are not fully understood.

Many studies have shown that some endogenous substances released during ischemic preconditioning activate a complex intracellular signaling cascade, involving mitochondrial potassium channels and mitochondrial permeability transition pores [7-9]. Mitochondria in cardiomyocytes provide ATP to the myocardium for survival and contraction. Mitochondria are also important sites of myocardial oxidative stress and calcium overload during ischemia-reperfusion [10]. Therefore, the mitochondrion is an important organelle for myocardial I/R injury. Some studies have shown that myocardial I/R injury, preconditioning, and conditioning affect the function of mitochondria [11-14]. Expression levels of some mitochondrial proteins are associated with myocardial I/R injury and ischemic preconditioning [15-17]. Ischemic conditioning and ischemic preconditioning exhibit similar mechanisms in myocardial protection [18-20]. Therefore, PPC may exert its myocardium pro-

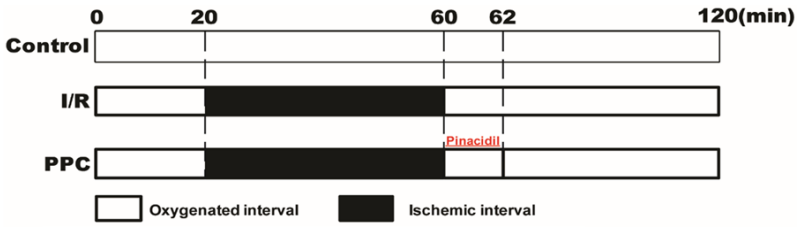

Figure 1. I/R injury and PPC perfusion protocol. Rats were allocated into three groups: control, I/R, and PPC. Rat hearts were equilibrated for 20 min before the application of the above protocols. Hearts were continuously perfused 100 min in the control group. Excluding the control group, all hearts were subjected to ischemia for 40 min (indicated with a black horizontal bar). The I/R group was perfused for 60 min. PPC hearts were perfused for 2 min with K-H solution containing pinacidil (50 μM), then perfused for 58 min.

tection effects via expression levels of some mitochondrial proteins.

This study was designed to detect expression levels of mitochondrial proteins in normal, I/R, and PPC hearts. Results may be useful in the exploration of potential biomarkers of I/R injury and PPC.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing 200-250 g, were purchased from the Center of Laboratory Animals in Third Military Medical University (Chongqing, China). Rats were housed in cages with ad libitum access to food and water. Room temperature was maintained at 24±1°C. All animals received humane care in compliance with the Guidelines for the Care and Use of Laboratory Animals in China (no. 14,924, 2001) and all experimental protocols were approved by the Zunyi Medical College Animal Care and Use Committee.

Materials

Sucrose, acrylamide, methylene bis-acrylamide, SDS, ammonium persulfate, glycerol, glycine, and mannitol were purchased from Amresco (Washington, USA). Nycodenz, EDTA, urea, thiourea, pinacidil, and TEMED were obtained from Sigma (Shanghai, China). DTT, BIO-Lyte, low melting point agarose, bromophenol blue, β-mercaptoethanol, iodoacetamide, CHAPS, protein quantification kits, IPG (immobilized pH gradient) strips, and PVDF were purchased from ad (California, USA). Anti-β-actin, anti-Calnexin, anti-GAPDH, and anti-COX IV were obtained from Santa Cruz (California, USA). Anti-ATPA, anti-NDUFA10, and anti-NAUFS2 were obtained from Abcam (Cambridge, UK). All other reagents were of analytical grade.

Perfusion protocol

Rats were anesthetized using intraperitoneal injections of sodium pentobarbital (40 mg/kg) and heparin (250 U/kg). Their hearts were rapidly excised and placed in cold K-H solution, with the following contents (in mM): 2.50 mM $CaCl_2$, 11.1 mM glucose, 1.19 mM KH_2PO_4, 4.75 mM KCl, 1.19 mM $MgCl_2 \cdot 6H_2O$, 118.00 mM NaCl, and 24.80 mM $NaHCO_3$, pH 7.40. Control, I/R, and PPC hearts were perfused, as previously reported [6, 14, 21], with the protocols displayed in **Figure 1**.

Eighteen isolated rat hearts were randomly divided into three groups (control, I/R, and PPC groups, n=6). Rat hearts were perfused with K-H solution for 20 minutes for equilibration. Control hearts were continuously perfused for 100 minutes. After equilibration, the hearts were subjected to 40 minutes of global ischemia. I/R hearts were perfused with K-H solution for 60 minutes. PPC hearts were perfused with K-H solution that contained pinacidil (50 μM) for 2 minutes, then re-perfused with K-H solution for 58 minutes. Data collection: Observation items were collected at T1 (average value during 1 minute at the end of equilibration) and T2 (average value during 1 minute at end of perfusion). Left ventricular end diastolic pressure (LVEDP) and left ventricular development pressure were recorded with a PowerLab 8/30 Data Acquisition and Analysis System (AD Instrument, Spain).

Mitochondria extraction

The purity of samples is the key to the study of mitochondrial proteomics. Mitochondria were prepared, as previously reported [14]. Briefly, hearts were cut into pieces and placed in ice-cold mitochondrion-separating medium (containing 210 mM mannitol, 700 mM sucrose, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.4). Left ventricular muscle tissue was cut under an ice

bath and homogenized (tissues). Thereafter, the tissue was centrifuged at 1,500 × g for 10 minutes. The supernatant was centrifuged at 12,000 × g for 10 minutes (crude mitochondria). Finally, Nycodenz density gradient medium was layered in a centrifuge tube, with the following concentration gradient: 34% 0.5 mL, 30% 0.8 mL, 25% 1.2 mL (containing the crude mitochondrial suspension), and 20% 0.3 mL. Centrifugation was conducted at 100,000 × g for 60 minutes to obtain purified mitochondria (purified mitochondria).

Electron microscopy was used to observe the quantity and integrity of purified mitochondria and crude mitochondria. Expression levels of β-actin (plasma membrane marker), calnexin (endoplasmic reticulum marker), GAPDH (cytoplasmic marker), and COX IV (mitochondrial inner membrane marker) were detected by Western blotting, confirming the purity of the samples. Equal amounts of total protein (40 μg) from each group were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes for immunoblotting. Primary antibodies were rabbit anti-β-actin, goat anti-calnexin, goat anti-GAPDH, and rabbit anti-COIV. Protein expression was visualized with horseradish peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) was detected using an Odyssey Infrared Imaging System (LI-COR, USA).

2-DE of mitochondrial proteins

The purified mitochondrial pellet was dissolved in hydration loading buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base, 1% DTT) for 10 minutes, then sonicated for 10 seconds. This was followed by centrifugation at 12,000 × g for 20 minutes to obtain mitochondrial proteins. 2-DE was performed, as described in the operation manual from Bio-Rad, with some necessary improvements.

A 24 cm (pH 5-8) IPG strip was rehydrated for 14 hours at 50 V in 500 mL of Hydration Sample Buffer, containing approximately 500 μg of the solubilized mitochondrial protein. IEF (isoelectric focusing) was carried out at 250 V for 1 hour as follows: 1,000 V for 3 hours, 4,000 V for 3 hours, and finally, 10,000 V increased incrementally to 80,000 V/h. The IPG strips were placed in 8 mL of equilibration solution (375 mM Tris-HCl, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, and 0.001% bromophenol blue), which contained 1.6% DTT during the first equilibration step and 2.5% iodoacetamide during the second equilibration step (14 minutes per equilibration step). Separation was performed using the Bio-Rad system. IPG strips were loaded onto a 12% SDS-PAGE gel. Running buffer (25 mM Tris, 192 mM glycine, 0.1% mM SDS, pH 8.3) was added and a constant current was applied for 16 hours. The gels were stained with silver nitrate. Stained gels were then scanned with an EPSON scanner.

Protein identification

PDQuest 8.0 was used to identify spots showing a more than two-fold difference between the control, I/R, and PPC groups, as previously reported [14]. Differentially-expressed protein particles were excised and gel-digested. The peptide mass fingerprint was obtained via MALDI-TOF-MS. The NCBInr protein database was searched with Mascot software. At the same time, peptide sequence information from two-stage tandem mass spectrometry was searched. Identification results were further confirmed.

Western blotting

Western blotting experiments were conducted with standard procedures [22]. In this study, expression levels of NDUFS2, ATPA, and NDUFA10 were detected via Western blotting. Western blotting data (normalized to COX IV) confirmed the reliability of 2DE and MALDI-TOF-MS results. Equal amounts of total protein (60 μg) from each group were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes for immunoblotting. Primary antibodies were rabbit anti-NDUFS2, goat anti-ATPA, goat anti-NDUFA10, and rabbit anti-COX IV. Protein expression was visualized with horseradish peroxidase-conjugated secondary antibodies (1:10000). Enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) was detected using an Odyssey Infrared Imaging System (LI-COR, USA).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed

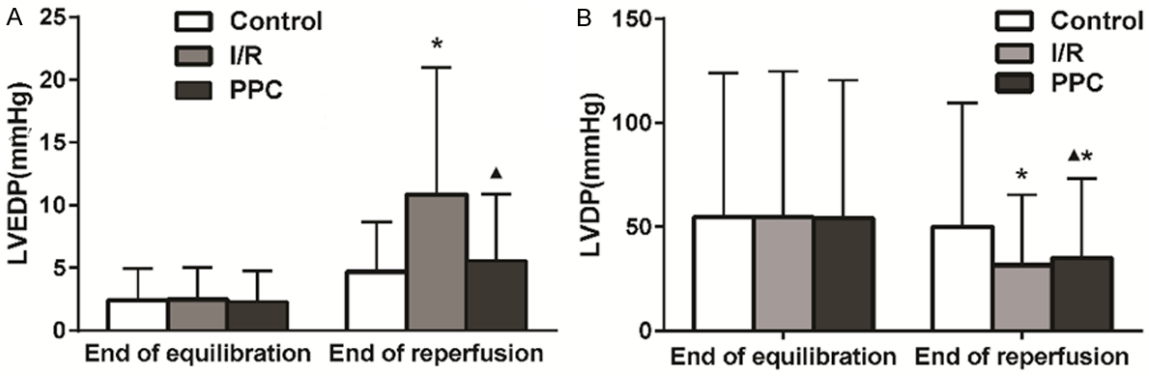

Figure 2. Left ventricular end diastolic pressure (LVEDP) were significantly elevated by I/R (A); Left ventricular developed pressure (LVDP) was significantly decreased in the I/R group and was higher in the control group than the PPC group (B). Data are presented as the mean ± S.D. *P<0.05 vs. control group; ▲P<0.05 vs. I/R group.

through two-way repeated-measures analysis of variance (ANOVA) or one-way ANOVA (SPSS for Windows version 17.0, Chicago, USA). If overall significance was found between the groups, a comparison was conducted using Student's unpaired *t*-test. P<0.05 indicates statistical significance.

Results

PPC improved cardiac function

LVEDP was lower in the PPC group and control group than in the I/R group (P<0.05, **Figure 2**). LVDP was higher in the control group than in the I/R group and PPC group (P<0.05, **Figure 2**). It was higher in the PPC group than in the I/R group (P<0.05). This result is consistent with previous results. In addition, previous works have shown that PPC significantly reduced myocardial infarct size and decreased mitochondrial damage (mitochondrial morphology, respiratory function) after I/R injury [4].

Purity identification of extracted and purified mitochondria

The quantity and integrity of purified mitochondria were significantly better than those of crude mitochondria evaluated through electron microscopy (**Figure 3A**, **3B**). Western blotting results revealed that expression levels of β-actin, calnexin, and GAPDH in purified mitochondria were significantly lower than those in heart tissue and crude mitochondria (P<0.05, **Figure 3C**, **3D**). The expression level of COX IV in purified mitochondria was higher than that in tissue and crude mitochondria (P<0.05, **Figure 3D**). Therefore, the purified mitochondria obtained in this study showed high purity and could be used for further experimentation.

I/R and PPC changed mitochondrial protein expression levels

The current study compared expression levels of mitochondrial proteins in control, I/R, and PPC groups using 2-DE. Images of the gels after silver nitrate staining are shown in **Figure 4** (A, control, B, I/R, C, PPC). Expression of four proteins changed more than two-fold between control and I/R groups, with one spot increasing and three spots decreasing (P<0.05, **Figure 4D**). Seven proteins were found to exhibit a two-fold change in the PPC group, with four spots increasing and three spots decreasing, compared with spots in the I/R group (P<0.05, **Figure 4E**).

Protein identification with MALDI-TOF-MS

Eleven spots were isolated from 2-DE gels of the I/R group and subjected to MALDI-TOF-MS. Peptide mass peaks were compared with those in the NCBI database. A description of the protein data is provided in **Table 1**.

PPC changed expression levels of NDUFS2, ATPA, and NDUFA10

Expression levels of NDUFS2 and NDUFA10 in control and PPC groups were lower than those in the I/R group (P<0.05, **Figure 5**). The expression level of ATPA in the control group was higher than that in I/R and PPC groups, while the PPC group showed higher ATPA expression than

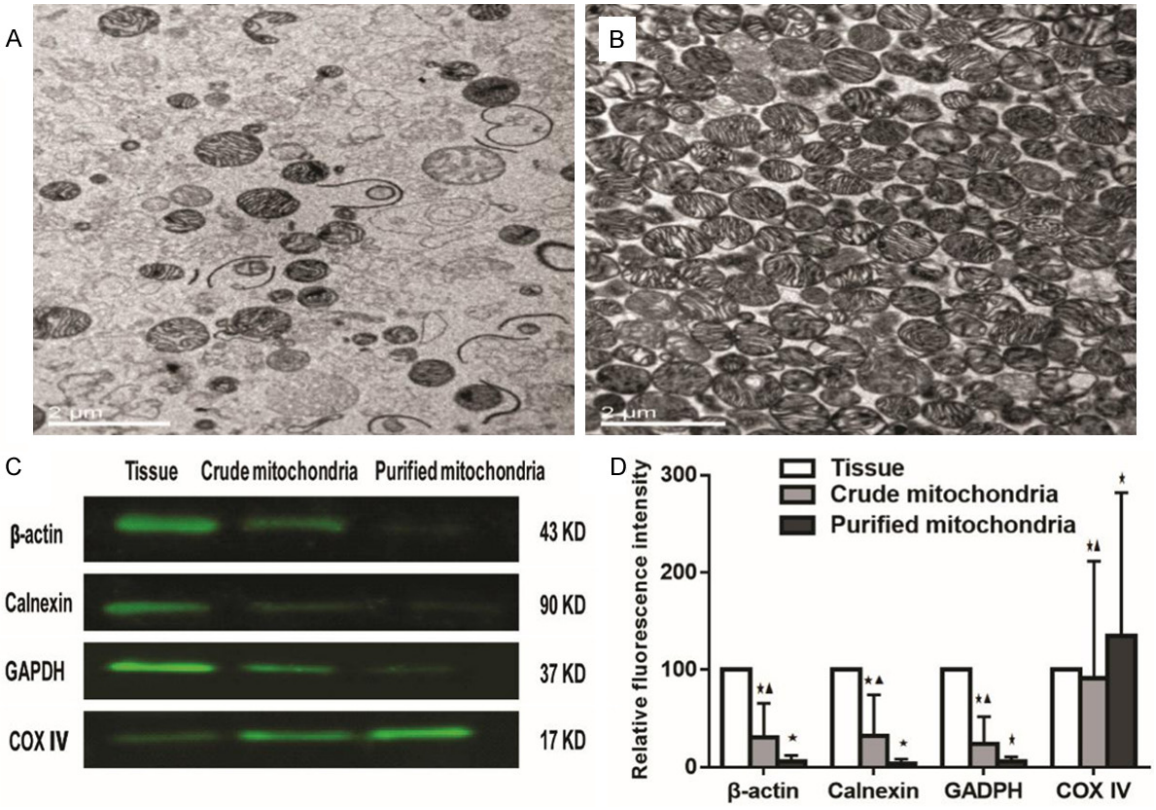

Figure 3. Confirmation of mitochondrion purity through electron microscopy and Western blotting. Representative electron microscopy images of crude mitochondria (A) and purified mitochondria (B). Pure mitochondria are collected with Nycodenz density gradient centrifugation method. Bar =2 μm. Western blotting results revealed that expression levels of β-actin, calnexin, GAPDH, and COX IV in purified mitochondria (C and D). Expression levels were significantly lower than those in heart tissue and crude mitochondria (P<0.05, C, D). The expression level of COX IV in purified mitochondria was higher than that in tissue and crude mitochondria (P<0.05, C, D). The fluorescence intensity of the tissue was set as 100. The fluorescence intensity of crude mitochondria and purified mitochondria was divided by the fluorescence intensity of the tissue and multiplied by 100 to obtain relative fluorescence intensity values. *P<0.05 vs. tissue; ▲P<0.05 vs. purified mitochondria (B, C).

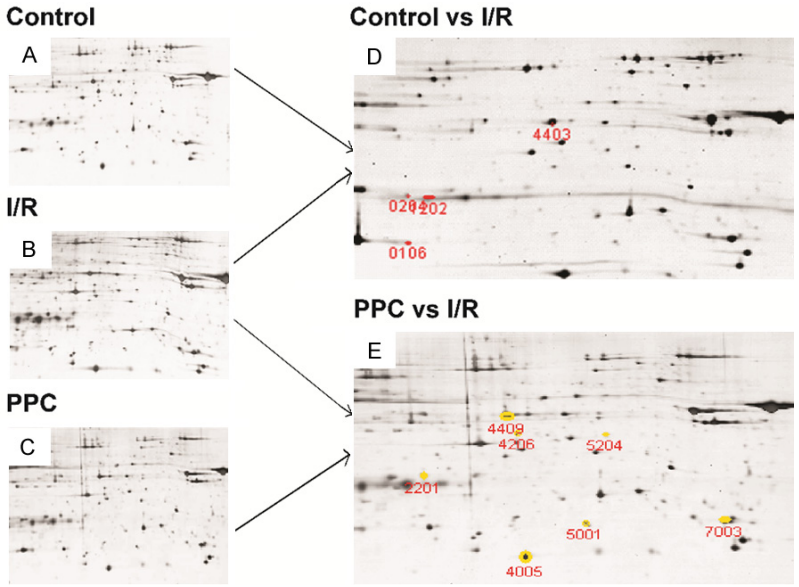

Figure 4. 2-DE analysis of mitochondrial proteins in rat hearts. Representative gels from the control (A), I/R (B), and PPC (C) groups. (D) Differentially expressed protein spots between the control and I/R groups. (E) Differentially expressed protein spots between the PPC and I/R groups.

the I/R group (P<0.05, **Figure 5**). Present results were consistent with 2-DE results, proving that the 2-DE results are reliable.

Discussion

Mitochondria are important sites of energy metabolism

Table 1. Differentially expressed proteins

Comparison	SSP	NCBI no.	AA% coverage	Protein ID	Mr	PI	Change >2 times
Con vs I/R	0106	gi|40538742	23%	ATPA	59831	9.22	↓
	1202	gi|13385006	47%	Isoform 2 of Cytochrome c1	29707	6.55	↓
	0204	gi|227500281	11%	ETFA	35272	8.62	↓
	4403	gi|58865384	26%	NDUFS2	52927	6.52	↑
PPC vs I/R	2201	gi|6015047	24%	ECH1	36491	8.13	↓
	4005	gi|9506411	31%	ATP synthase subunit d	18809	6.17	↑
	4206	gi|170295834	50%	NDUFA10	40753	7.64	↑
	4409	gi|58865384	53%	NDUFS2	52927	6.52	↑
	5001	gi|9506411	31%	ATP synthase subunit d	18809	6.17	↓
	5204	gi|16758446	32%	IDHA	40044	6.47	↓
	7003	gi|51092268	39%	NDUFV2	27703	6.23	↑

SSP: Protein spot numbers given by PDQues; NCBI no. accession number of the NCBI database protein; AA% coverage: amino acid sequence match rate.

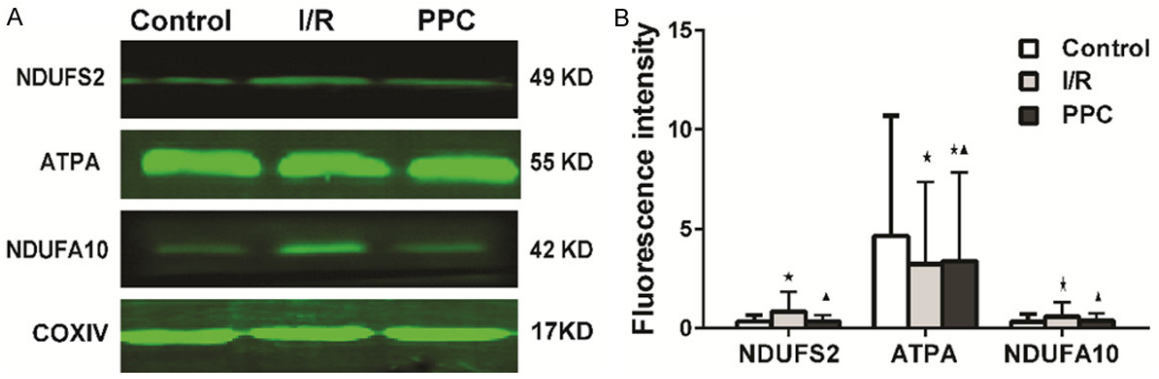

Figure 5. Protein expression of NDUFS2, ATPA, and NDUFA10. Expression levels of NDUFS2 and NDUFA10 were lower in the control and PPC groups than in the I/R group (P<0.05). The expression level of ATPA in the control group was higher than in the I/R and PPC groups, and its expression was higher in the PPC group than in the I/R group (P<0.05). ★P<0.05 vs. the control; ▲P<0.05 vs. I/R.

in eukaryotic cells. Myocardial tissue is particularly sensitive to changes in the structure and function of the mitochondria. In this study, it was found that I/R seriously deteriorated cardiac function, while PPC significantly improved cardiac function in Langendorff rat hearts. Comparative proteomics can provide reliable data for exploring these issues regarding protein expression on different molecular levels [23, 24]. Studies have shown that mito-KATP opening may be the trigger point and end-effector of myocardial protection [25]. PPC exerts obvious protective effects against myocardial I/R injury, which is bound to cause some changes in mitochondrial protein expression. Therefore, comparative proteomic analysis of rat myocardial mitochondria was conducted to detect potential biomarkers of I/R injury and PPC. There were 4 differentially-expressed proteins identified between normal and I/R hearts and 7 differentially-expressed proteins between PPC-treated hearts and I/R hearts. These proteins are associated with the mitochondrial respiratory chain and energy metabolism. They could be potential biomarkers for hearts subjected to I/R injury and receiving PPC.

NADH dehydrogenase

The current study found that expression of NDUFS2 increased after I/R injury. In addition, expression levels of NDUFS2, NDUFA10, and NDUFV2 decreased in PPC hearts, compared with I/R hearts. The NADH dehydrogenase sub-

unit constitutes the catalytic core of the complex I and NDUFS2 is also one of its catalytic cores [26]. The underlying mechanisms may be that I/R injury leads to inhibition of mitochondrial oxidative stress and increased production of hydrogen ions, which causes intracellular metabolic acidosis. It was hypothesized that mito-KATP opens, activating the NADH dehydrogenase respiration chain, reducing ROS production, increasing ATP production, accelerating the exchange of H^+-K^+ and H^+-Na^+, and ameliorating intracellular and mitochondrial acidosis. These effects are associated with changes in expression levels of 3 proteins. Mitochondrial proteomic analysis of myocardial ischemic preconditioning in rabbits showed that expression of NDUFA10 and NDUFS2 was lower after ischemic preconditioning. The authors speculate that these changes may be considered a compensatory measure to protect mitochondrial function [15].

ATP synthase

In this study, two subunits of ATP synthase were identified, ATPA and ATPD. Expression levels were relatively decreased in I/R hearts. Interestingly, two protein spots were identified as ATPD. One of these spots was upregulated, while the other downregulated. It was speculated that PPC causes phosphorylation of ATPD and that this modification plays an important role in the mechanisms of mito-KATP opening in myocardial protection. Studies have shown that I/R leads to severe inhibition of ATP synthase and a significant reduction in ATP production [27, 28]. ATP synthase plays an important role in mito-KATP opening in myocardial protection mechanisms [29, 30]. Researchers have discovered that pretreatment with adenosine and diazoxide in a myocardial mitochondrial proteomic analysis caused phosphorylation of ATP synthase, which could protect the myocardium from I/R injury [31]. Studies have also shown that phosphorylation of an ATP synthase subunit may be related to the cardioprotective mechanisms of mito-KATP opening [32, 33].

Other proteins

This study also showed that expression levels of isoform 2 of cytochrome c1 decreased when hearts suffered I/R injury and that expression levels of IDHA and ECH1 decreased when hearts received PPC treatment. These proteins are associated with the mitochondrial respiratory chain and energy metabolism. Reports on these proteins associated with myocardial ischemia-reperfusion and PPC are quite rare.

Proteomic analysis of mitochondrial proteins in control, I/R, and PPC rat hearts provided an effective approach for elucidating the molecular mechanisms of I/R injury, as well as the protective effects of PPC. Eleven protein spots with differences in abundance were found through 2-DE. These differentially-expressed proteins were identified via MALDI-TOF MS. These proteins may be potential biomarkers of I/R and PPC hearts. The current study had some limitations, however. It was speculated that ATPD carries out phosphorylation after PPC treatment, but this has not been confirmed experimentally. Additionally, this study used narrow pH (5-8) IPG strips, which may have resulted in some undetected proteins and may be the reason that only eleven differentially-expressed proteins were found.

Acknowledgements

This work was supported by grants from Special Scientific Research Fund for Public Welfare, Ministry of Health of China (Grant No. 20080-2173), and the National Natural Science Foundation of China (Grant No. 8176020318).

Disclosure of conflict of interest

None.

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