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**A proteomic study reveals Shengmai injection preventing cardiac ischemia-reperfusion injury via energy metabolism modulation**

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## Abstract

Energy metabolism modulation plays an important role in protecting heart from ischemia-reperfusion (IR) injury. Shengmai injection (SMI) is a Chinese medicine widely used in China to treat ischemic heart diseases with speculated functions of modulating energy metabolism. To uncover the molecular mechanisms underlying the cardioprotective activity of SMI with the modulation of energy metabolism, a proteomic analysis was performed on ischemia-reperfusion (IR) injury heart of rats in this study. Two-dimensional gel electrophoresis (2-DE) was used to measure the protein expression profiles of heart tissues. Differentially expressed proteins among groups were identified using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS/MS). Western blot analysis was used to validate differentially expressed proteins. Proteomic data revealed 14 major differentially expressed proteins that are related with energy metabolism. It was found that the glucose oxidation, TCA cycle and ATP synthesis related proteins were consistently up-regulated in SMI treated rats, which is beneficial to aerobic respiration and ATP generation. On the contrary, two proteins catalyzing fatty acid  $\beta$ -oxidation were down-regulated implying the inhibition of this pathway to avoid high oxygen consumption. It is thus concluded that one of the major mechanisms of SMI's protection against IR injury was modulation of myocardial energy metabolism to improve cardiac efficiency through multiple metabolic pathways including stimulating glucose metabolism and inhibiting fatty acid metabolism. It provided potential protein targets for the therapeutic strategy through modulation of myocardial energy metabolism.

## Introduction

Ischemic heart disease is one of the leading causes of morbidity and mortality in industrialized countries. Although reperfusion therapy can effectively treat ischemic injury, severe side effects such as cardiac over-contractile function, arrhythmia, myocardial stunning and myocardial infarction often occur during the process.<sup>1, 2</sup> Ischemia-reperfusion (IR) injury is one of the most pivotal pathological factors causing the side effects. The major mediators of IR injury include reactive oxygen species (ROS), dysregulation of intracellular  $\text{Ca}^{2+}$  overload and reperfusion arrhythmia.<sup>1, 3</sup> They are all related to ATP depletion due to cardiac energy metabolism dysfunction.<sup>4-6</sup> The modulation and optimization of myocardial energy metabolism to improve cardiac efficiency thus represents a promising area for cardiac therapeutic interventions.<sup>7, 8</sup>

Shengmai injection (SMI), a Chinese medicine comprising *Panax ginseng*, *Ophiopogon japonicas* and *Schisandra chinensis*, is widely used in China for the treatment of cardiac emergencies, such as coronary atherosclerotic cardiopathy, viral myocarditis and myocardial infarction.<sup>9-11</sup> Clinical studies have demonstrated that SMI can improve cardiac function of patients with dilated cardiomyopathy<sup>12</sup> and inhibit the inflammatory reaction in patients with acute coronary syndrome.<sup>13</sup> SMI was also reported to have protective effects against IR injury in isolated rabbit hearts through the improvement of cardiac energy metabolism and scavenging oxygen radical.<sup>14</sup> Our previous pharmacological study has demonstrated SMI's cardioprotective effects against IR injury by reducing cell death, ROS and inflammation related factors such as  $\text{TNF-}\alpha$ 、 $\text{IL-1}\beta$ 、 $\text{IL-6}$ 、 $\text{IL-8}$  in IR rats.<sup>15</sup> However, the molecular mechanisms of SMI underlying its pharmacological activity remain unknown.

Proteomics is an efficient approach for measurement of protein expressions in different states and it has been successfully used to study mechanisms of traditional Chinese medicine.<sup>16-20</sup> To further uncover the molecular mechanisms underlying the myocardial protection of SMI with the modulation of energy metabolism, a proteomic

analysis with two-dimensional gel electrophoresis (2-DE) and matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS/MS) techniques was performed.

## Materials and methods

### Chemicals and reagents

Shengmai injection was supplied by SZYY Group Pharmaceutical Limited (Jiangyan, Jiangsu, China). Bisacrylamide, acrylamide, Tris (hydroxymethyl) aminomethane (Tris), sodium dodecyl sulfate (SDS), glycine, N,N,N',N'-tetramethylethyldiamide (TEMED), ammonium persulfate (AP), glycerol, carbamide (urea), bromophenol blue, 1,4-dithiothreitol (DTT), 3-(3-dimethylammonio)propanesulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), sodium carbonate, agarose and sodium thiosulfate were purchased from Shanghai Biotech Co., Ltd. (Shanghai, China). Sodium acetate, silver nitrate and iodoacetamide were purchased from Hangzhou HaoTian Biotechnology Co., Ltd. (Hangzhou, China). Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). Protease inhibitor cocktail was purchased from Amresco (Solon, OH). 2D-Quant kit was obtained from GE Healthcare (Amersham, Freiburg, Germany). All other solvents were of analytical grade.

### Component analysis of SMI

To illustrate chemical composition of SMI, the quantitative analysis of its major components and their pharmacokinetics study in rats has been performed in our laboratory.<sup>21</sup> The result indicated ginsenosides in *Panax ginseng*, lignan in *Schisandra chinensis* and ophiopogonin in *Ophiopogon japonicas* were major components of SMI, which conformed to many reports about chemical materials studies of SMI.<sup>22-24</sup> The chemical structures and concentrations of major components in SMI were listed in Table 1 (shown in electronic supplementary information).

### Animals and treatments

Male Sprague–Dawley (SD) rats, weighting 220–250g, were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were maintained under standard laboratory condition with temperature at  $22 \pm 2$  °C, relative humidity at  $50 \pm 10\%$ , photo period (12-h dark/12-h light) and were fed normal diet and water ad libitum. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Ischemia-reperfusion injury was produced by occlusion of the left anterior descending coronary artery and then reperfusion. Briefly, rats were anaesthetized by intraperitoneal injection with 5% chloralhydrate (6mL/kg). The pericardium was opened to expose the heart. The left arterial descending coronary artery was ligated by a 6/0 silk suture passed through a small vinyl tube. After 30 min occlusion (ischemia), the myocardium was reperfused by cutting the silk suture. Before and during the operation, electrocardiogram of each rat was recorded to make sure that the ischemia was induced. In sham-operated rats, the ligation suture was not placed in the heart. Rats in IR+SMI group were given Shengmai injection 4 days before operation and continued for 7 days with the dose of 6mL/kg/d. Rats in sham and IR groups were administered with 0.9% normal saline in the same manner.

### **Sample preparation**

Rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg) at 8 h after the treatment and then bled from inferior vena cava. After removal of blood, hearts were harvested for protein extraction. For protein extraction, the fresh hearts were quickly removed and perfused for 1–2 min with cold modified Krebs–Henseleit solution (200mM sucrose, 200mM Tris–HCl, 0.4mM CaCl<sub>2</sub>, pH7.0). After removing fat and connective tissues, the left ventricle was snap frozen in liquid nitrogen and stored at  $-80$  °C. To obtain protein extracts, 100 mg tissue was homogenized in 1mL of lysis buffer (9M urea, 2% (w/v) CHAPS, 0.5% (w/v) DTT, protease inhibitor cocktail) at 4 °C. The resulting homogenate was centrifuged for 10min at 10,000g and then supernatant was collected. Protein concentration of sample

was measured using 2D-Quant Kit.

### **Two-dimensional electrophoresis and image analysis**

2-DE was performed as previously reported with minor modification.<sup>16</sup> Briefly, 80 $\mu$ g proteins were applied to a 24 cm non-linear gradient IPG-strips, pH 3–10 (Amersham Biosciences). Strips were active during rehydration (rehydration solution: 2% CHAPS, 0.5% immobilized pH gradient (IPG) buffer, 0.02% bromophenol blue, 8 M urea, 2.8 mg/mL DTT) for 12 h at 30 V, followed by focusing for 1 h at 100 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 30min for voltage increasing to 8000 V, and remaining 8000 V for 73 kVh on an IPGPhor (Amersham Biosciences). Focused IPG strips were equilibrated twice for 15 min with gentle shaking in equilibration buffer (30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue, 50 mM Tris-HCl buffer (pH 8.8), 6M urea and 10 mg/mL DTT). In the second equilibration buffer, DTT was replaced with 25mg/mL iodoacetamide. After equilibration, the IPG strips were gently rinsed with SDS electrophoresis buffer to remove excessive buffer and then applied onto a 12% polyacrylamide gradient gel (200 $\times$ 260 $\times$ 1 mm<sup>3</sup>) overlaid with agarose gel (0.5% agarose and 0.002% bromophenol blue in a standard SDS running buffer). The second dimension separation was performed sequentially with a constant voltage of 5W/gel for 1h, followed by 20W/gel for 6h using the Ettan DALTsix electrophoresis system (Amersham Biosciences) until bromophenol blue reached the bottom of the gel. After SDS-PAGE, the separated gels were visualized by silver staining.

The silver-stained gels were scanned using a Powerlook 2100XL scanner ( $U_{\max}$ , Hanchu, Taiwan). The gel images were analyzed using the ImageMaster 2D Platinum software (version 5, Amersham Biosciences, Piscataway, NJ). The intensity of the spot (%) represents the percentage of a protein in the total proteins. The most promising candidate differential spots were then selected for excision from a preparative gel for further identification by MALDI-TOF-MS/MS.

### **MALDI-TOF-MS/MS for protein identification**

Protein spots were excised from the gels and placed into a 96-well microtitre plate.

Gel pieces were destained with a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1:1) for 20 min at room temperature. Then they were washed twice with deionized water, shrunk by dehydration in ACN. The samples were then swollen in a digestion buffer containing 20 mM ammonium bicarbonate and 12.5 ng/ $\mu$ L trypsin at 4°C. After 30 min incubation, the gels were digested more than 12 h at 37°C. Peptides were then extracted twice using 0.1% trifluoroacetic acid (TFA) in 50% ACN. The peptide solution described above was dried under the protection of N<sub>2</sub>, mixed with 0.8  $\mu$ L matrix solution (5mg/ml  $\alpha$ -cyano-4-hydroxy-cinnamic acid diluted in 0.1%TFA, 50%ACN). Then the mixture was spotted on a MALDI target plate (AB SCIEX). MS analysis of peptide was performed on an AB SCIEX 5800 TOF/TOF. The UV laser was operated at a 400 Hz repetition rate with wavelength of 355 nm. The accelerated voltage was operated at 20 kV. Myoglobin digested with trypsin was used to calibrate the mass instrument with internal calibration mode. All acquired spectra of samples were processed using TOF/TOF Explorer™ Software (AB SCIEX) in a default mode. The data were searched by GPS Explorer (V3.6) with the search engine MASCOT (V2.3) to screen the NCBI protein sequence database. The search parameters were as follows: the database rat p (113333 sequences; 46339805 residues), trypsin digest with one missing cleavage, MS tolerance was set at 100 ppm, MS/MS tolerance of 0.6 Da. All the identified proteins should have a protein score more than 63 ( $P < 0.05$ ).

#### **Validation with Western blot analysis**

Aldose reductase (AKR1B1), Aldolase A, Short-chain specific acyl-CoA dehydrogenase (ACADS), 3-ketoacyl-CoA thiolase (ACAA2), Malate dehydrogenase (MDH2) and ATP synthase, H<sup>+</sup> transporting (ATP5A) were validated with Western blot analysis. The protein of  $\beta$ -Actin was used as an internal control. Twenty micrograms of cardiac protein was separated by NuPAGE® 10% Bis-Tris gels using electrophoresis, and electrotransferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). Blots in the membranes were blocked for 2 h with 5% BSA in Tris-buffered saline (TBS) at room temperature and

then incubated overnight at 4°C with primary antibodies: mouse anti-AKR1B1 (1:1000), rabbit anti-Aldolase A(1:1000), rabbit anti-ACADS (1:1000), mouse anti-ACAA2 (1:500), mouse anti-MDH2 (1:1000), and rabbit anti-ATP5A (1:2000) (Abcam, Hong Kong). The membranes were then washed with 0.1% TBS-Tween 20 and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at a 1:10,000 dilution for 1 h at room temperature. After several washes, immunoreactive proteins were visualized with SuprerSignal West Femto Maximum Sensitivity Substrate (Thermo Corporation) and captured on ChemiDox XRS gel imaging system (Bio-Rad). Protein levels were quantitated using Quantity One software (Bio-Rad).

### **Statistical analysis**

Data were expressed as Mean  $\pm$  SD. Difference between groups was analyzed by Student's *t*-test. The *P*-values less than 0.05 were considered to be statistically significant. Multiple testing correction was performed using false discovery rate analysis with the function `mafdr.m` in Matlab.

## **Results**

### **Effect of SMI on left ventricular ejection fraction (LVEF %)**

To evaluate cardioprotective effects of SMI, left ventricular ejection fraction (LVEF%) of rats were measured by echocardiography. The result of LVEF % has been declared in our previous report<sup>15</sup> and reproduced in Fig. 1. It was found that LVEF % of IR group was significantly reduced comparing to sham group. Treatment with SMI resulted in a marked recovery in LVEF % of IR group. This result indicated that SMI can effectively improve cardiac function of rats with IR injury.

### **Differential proteins analysis**

Representative images of 2-DE gel presenting sham, IR and IR+SMI groups were

shown in Fig. 2. Image analysis with ImageMaster 2D Platinum revealed that an average of 790, 876 and 809 protein spots were detected from sham, IR and IR+SMI groups, respectively. Most of the protein spots were distributed in the region of isoelectric point (pI) 5–9 and had molecular weight between 20 and 60 kDa. Compared with IR group, 14 protein spots were found to be significantly altered in SMI treated group. Out of these proteins indicated by the arrows in Fig. 2(A), 11 proteins were up-regulated and 3 proteins were down-regulated. For all the selected spots, the relative spot intensity (%) of the proteins in IR and IR+ SMI group was calculated, using sham group as reference (100%). The significance of the changes in protein abundance was calculated by student *t*-test and corrected by false discovery rate analysis. To illustrate the change in intensity of the protein spots, some representative enlarged 2D gel images are shown in Fig. 3.

The differentially expressed protein spots were isolated from the 2D gel and subjected to trypsin digestion and identified successfully by MALDI-TOF-MS/MS. The peptides mass peaks were compared with those in the NCBI database. The complete list of protein data identified by MS/MS was listed in Table 2 (shown in electronic supplementary information). Furthermore, the mass spectrum of short-chain specific acyl-CoA dehydrogenase protein is shown in Fig. 4 as a representative result of MALDI-TOF-MS/MS analysis of the 14 protein spots. We found that most differential proteins were related to energy metabolism including glucose metabolism, lipid metabolism, TCA cycle and respiratory chain (Rsp-chain). In addition, three other proteins (cytokeratin-6A, Tu translation elongation factor and rCG27551) were also detected.

### Western blot validation

The immunoblotting data from Western blot was shown in Fig. 5. From the results, the AKR1B1, Aldolase A, ATP5A and MDH2 levels in IR+SMI group increased comparing to those in IR group ( $P<0.01$ ). The levels of ACAA2 and ACADS decreased in IR+SMI group comparing to the IR group ( $P<0.05$ ). These results confirmed the findings from 2-DE gel data shown in Fig. 3.

## Discussion

Cardiac efficiency and function is typically reduced in IR injury due to perturbation and dysfunction of cardiac energy metabolism which results in ATP resynthesis disruption and depletion. Lack of ATP is well accepted as an essential episode in IR injury, which emerges during ischemia, and remains over the reperfusion.<sup>25</sup> Under normal physiological conditions, carbohydrates (i.e. glucose and lactate) and fatty acids are used as primary metabolic fuels to produce 95% ATP to sustain cardiac functions. These two energy substrate metabolism are tightly regulated and cardiac pathological states of IR cause perturbations of this myocardial energy substrate metabolism. Decrease of glucose metabolism and increase of lipid metabolism has been found contributing to the progression of myocardial injury.<sup>26,27</sup>

In this study, 11 out of 14 differentially expressed proteins were associated with cardiac energy metabolism. We found that five glucose and lactate oxidation related proteins including triosephosphate isomerase, pyruvate dehydrogenase E1 component subunit beta, aldose reductase, aldolase A and L-lactate dehydrogenase B chain were down-regulated in IR injury heart when compared to sham-operation heart. On the contrary, two fatty acid  $\beta$ -oxidation related proteins, short-chain specific acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, were found up-regulated. These results validated the inhibition of carbohydrate oxidation and the stimulation of fatty acid  $\beta$ -oxidation in post-ischemic and reperfusion period at enzymic protein level. So our study provided proteomic evidence for the metabolic change of glucose and fatty acid during IR as reported before.<sup>28-31</sup> Fatty acid  $\beta$ -oxidation generates the same amount of ATP at the expense of higher oxygen requirement than carbohydrate oxidation, which makes fatty acid a less efficient substrate for ATP synthesis. The shift of energy substrate metabolism towards fatty acid contributes to the reduction of cardiac efficiency by decreasing the efficiency of ATP generation in IR injury heart. Therefore, pharmacologic agents to reverse this shift by directly stimulating glucose metabolism or indirectly by inhibiting fatty acid metabolism have been explored for

their potential cardioprotective effects in treating ischemic heart diseases. For example, dichloroacetate has the effect of increasing pyruvate dehydrogenase expression (rate-limiting enzyme of glucose oxidation) to stimulate glucose oxidation and has been shown to dramatically improve recovery of mechanical function after ischemia.<sup>30, 32</sup> On the other hand, trimetazidine also has anti-ischemic properties by targeting 3-ketoacyl-CoA thiolase to inhibit fatty acid metabolism.<sup>33, 34</sup> Interestingly, it was found in this study that SMI treatment modulated several proteins e.g. pyruvate dehydrogenase and 3-ketoacyl-CoA thiolase in the right direction simultaneously. Thus significant up-regulation of the carbohydrate oxidation as well as down-regulation of fatty acid  $\beta$ -oxidation was achieved by a multi-point systemic regulation to recover the efficiency of aerobic metabolism and ATP generation by SMI (Fig. 6).

TCA cycle is a series of enzyme-catalyzed chemical reactions that form a key part of aerobic respiration in cells and the major path of ATP yield. In this study, malate dehydrogenase (MDH), an important enzyme in TCA cycle catalyzing malic acid oxidation was found to be down-regulated in IR injury heart, which suggested TCA cycle was inhibited after IR injury due to the deficient oxygen supply. It was observed that MDH was up-regulated after SMI treatment (Fig. 6). This result may be explained by that SMI improved coronary microcirculation in IR injury rats due to the increased release of NO which had been reported in our previous study that SMI could improve NO release in rats of myocardial ischemia.<sup>35</sup> Moreover, the effect of improved NO release may be contributed by its main components of ginsenosides which exert vasorelaxant effect through the activation of NO in endothelial cells.<sup>36-38</sup> Thus, the increased level of TCA cycle related enzyme found in this study may be attributed for NO-induced vasorelaxant effect and the partly resumed oxygen delivery. Eventually, more energy would be obtained through the improvement of the mitochondrial aerobic respiration. The modulation of TCA cycle to improve cardiac energy metabolism could be another beneficial direction in ischemic heart diseases.<sup>16,</sup>

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In addition, three respiratory chain-related proteins, ATP synthase, H<sup>+</sup> transporting,

ATP synthase subunit beta and creatine kinase M-type were indentified in this study. Creatine kinase (CK) catalyzes the reverse transformation of high-energy phosphate bond between creatine and ATP, and high level of CK often indicates myocardial cell injury.<sup>40</sup> The down-regulation of CK induced by SMI treatment suggests the protective effect of SMI against myocardial cell injury from IR. ATP synthase catalyzes mitochondrial oxidative phosphorylation, coupling with oxidizing reduced NADH and FADH<sub>2</sub> from TCA cycle to produce ATP. Lower expression of ATP synthase in IR injury heart indicated ATP synthesis disorder, and it was found SMI treatment can recover ATP synthesis by significantly up-regualting the expression of ATP synthase in this study (Fig. 6). This result demonstrated more energy recover after SMI treatment in IR injury rats.

In summary, the result of this study indicated SMI can modulate multiple pathways of myocardial energy metabolism including: stimulation of glucose metabolism, inhibition of lipid metabolism, enhancement of TCA cycle and ATP synthesis, as summarized in Fig. 6. The overall effect is beneficial to optimizing energy production and improvement of cardiac efficiency. Thus, our proteomic study revealed one of the major molecular mechanisms of SMI's actions against heart tissues injury caused by ischemia-reperfusion. This study also provided evidence for the therapeutic strategy of the modulation and optimization of myocardial energy metabolism to improve cardiac efficiency for therapeutic intervention in ischemic heart diseases.

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## Figure Captions

**Fig. 1.** Effect of SMI on LVEF % in IR injury rats, data are expressed as the Mean  $\pm$  SD ( $n=10$ ). \*\*  $P<0.01$  vs. Sham group, #  $P<0.05$  vs. IR group.

**Fig. 2.** Representative 2-DE gel images obtained from heart protein extracts from (A) sham-operated, (B) IR and (C) SMI treated rats. Differential proteins were marked by arrow and number.

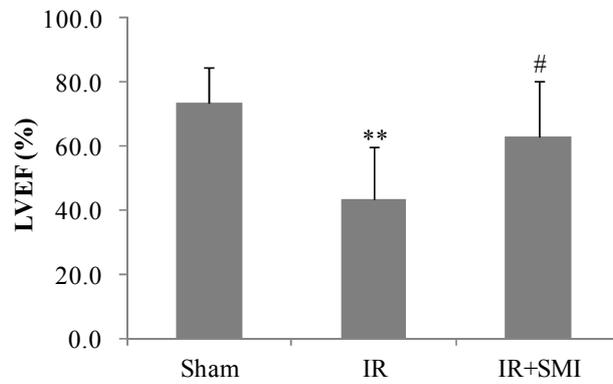
**Fig. 3.** Some examples of differential proteins in sham, IR and IR+SMI groups, (A) Representative enlarged protein spots. (B) Bar graph comparing mean values in sham, IR and IR+SMI groups (% to sham). Data are expressed as the Mean  $\pm$  SD ( $n=5$ ). \*  $P<0.05$ , \*\*  $P<0.01$  vs. Sham group, #  $P<0.05$ , ###  $P<0.01$  vs. IR group. All five proteins show FDR $<0.05$  for both comparisons.

**Fig. 4.** The result of the MALDI-TOF-MS/MS analysis of the protein spot 6. By searching NCBI protein sequence database, it was identified to be short-chain specific acyl-CoA dehydrogenase (NCBI accession number 11968090). (A) Peptide mass fingerprint of the tryptic digest of spot 6. Peptide signals identified was marked with asterisks. (B) MS/MS profile of the peptide with a mass of 1538.76 Da. y-ions and b-ions resulting from fragmentation of the peptides and amino acids are indicated.

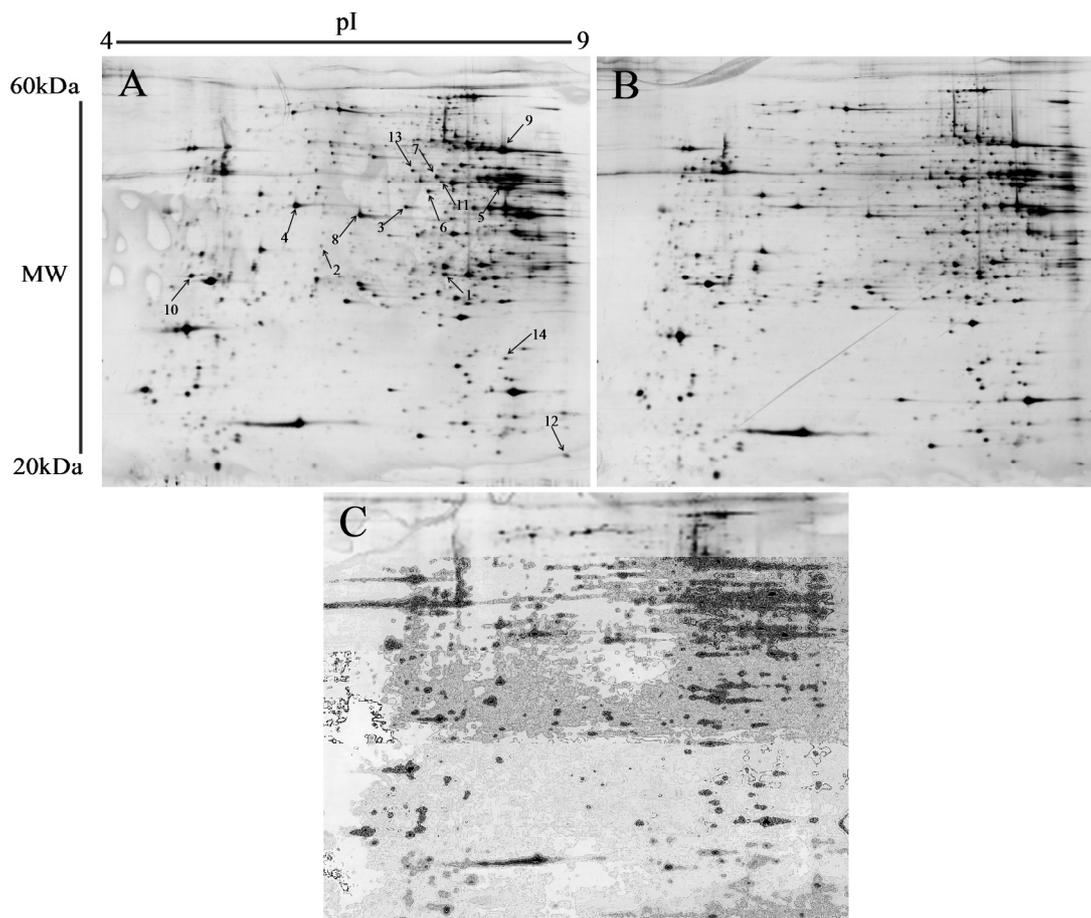
**Fig. 5.** Western blot analysis of Aldose reductase (AKR1B1), Aldolase A, ATP synthase, H<sup>+</sup> transporting (ATP5A), Malate dehydrogenase (MDH2), 3-ketoacyl-CoA thiolase (ACAA2), Short-chain specific acyl-CoA dehydrogenase (ACADS) and their corresponding  $\beta$ -Actin in sham, IR and IR+SMI groups. (A) Representative immunoblot. (B) Bar graph comparing mean values in sham, IR and IR+SMI groups (% to sham). Data are expressed as the Mean  $\pm$  SD ( $n=5$ ). \*  $P<0.05$ , \*\*  $P<0.01$  vs. Sham group, #  $P<0.05$ , ###  $P<0.01$  vs. IR group. The validated proteins were matched with spots of differential proteins in Fig. 3.

**Fig. 6.** Schematic depiction of myocardial energy metabolism together with modulation by SMI treatment; G-6-P, Glucose-6-phosphate; F-6-P, Fructose-6-phosphate; F-1,6-BP, Fructose-1,6-bisphosphate; G3P,

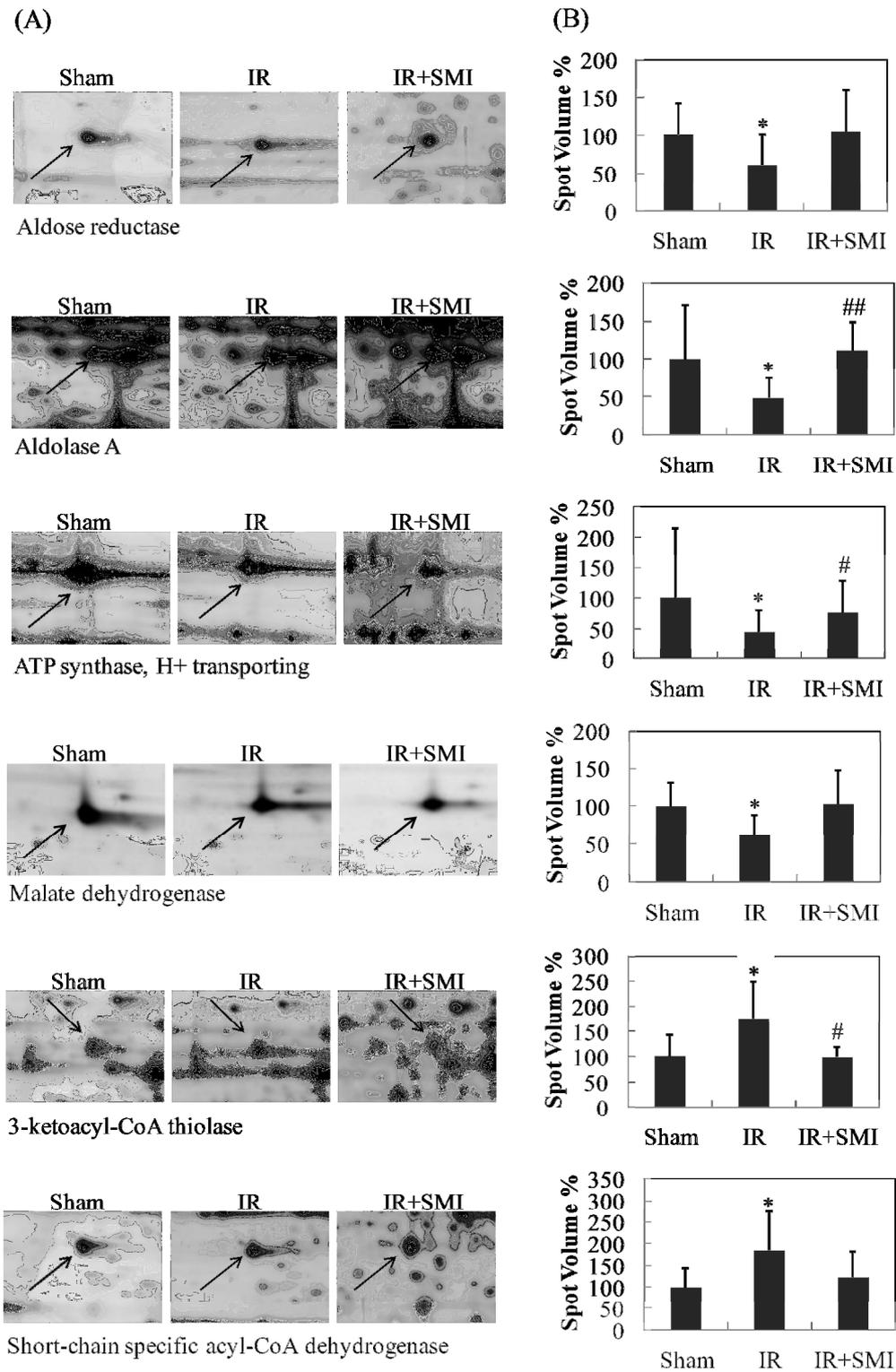
Glyceraldehyde-3-phosphate; SDH, Sorbitol dehydrogenase; DHAP, Dihydroxyacetone phosphate; TCA, Tricarboxylic acid



**Fig. 1.** Effect of SMI on LVEF % in IR rats, data are expressed as the Mean  $\pm$  SD ( $n=10$ ). \*\*  $P<0.01$  vs. Sham group, #  $P<0.05$  vs. IR group

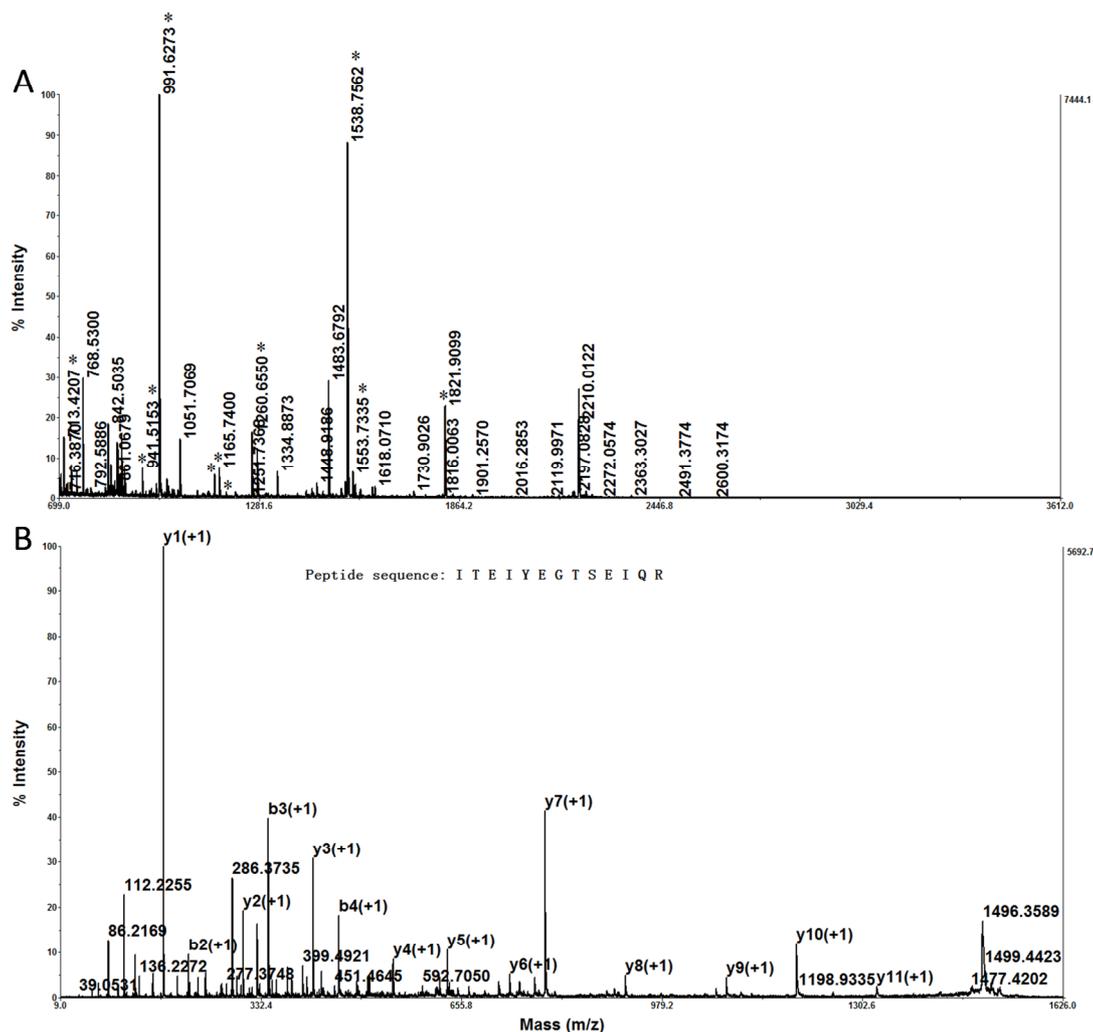


**Fig. 2.** Representative 2-DE gel images obtained from heart protein extracts from (A) sham-operated, (B) IR and (C) SMI treated rats. Differential proteins were marked by arrow and number.

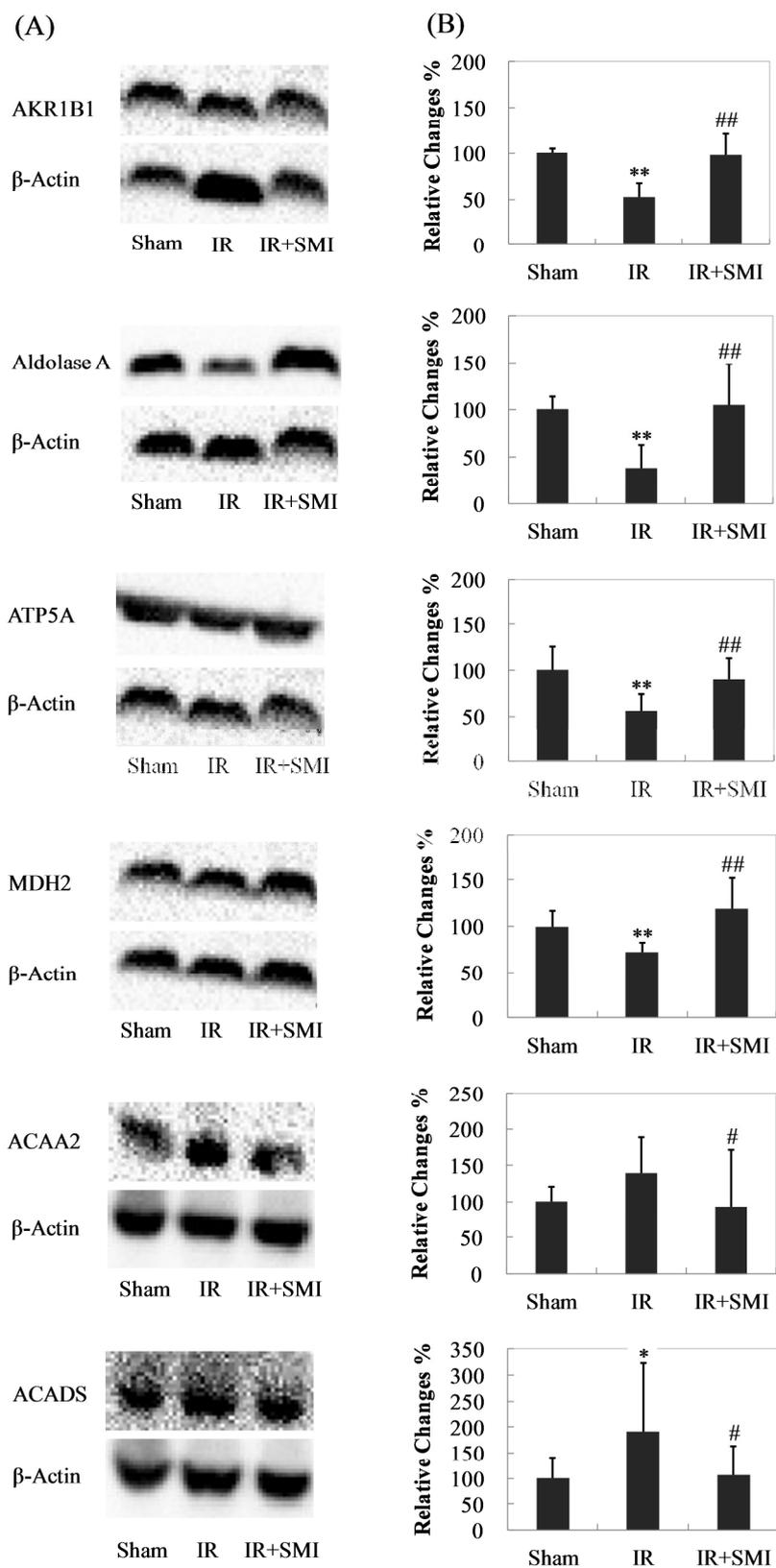


**Fig. 3.** Some examples of differential proteins in sham, IR and IR+SMI groups, (A) Representative enlarged protein spots. (B) Bar graph comparing mean values in sham, IR and IR+SMI groups (% to sham). Data are expressed as the Mean  $\pm$  SD ( $n=5$ ). \*  $P<0.05$ , \*\*  $P<0.01$  vs. Sham group, #  $P<0.05$ , ##  $P<0.01$  vs. IR group. All five

proteins show FDR<0.05 for both comparisons.

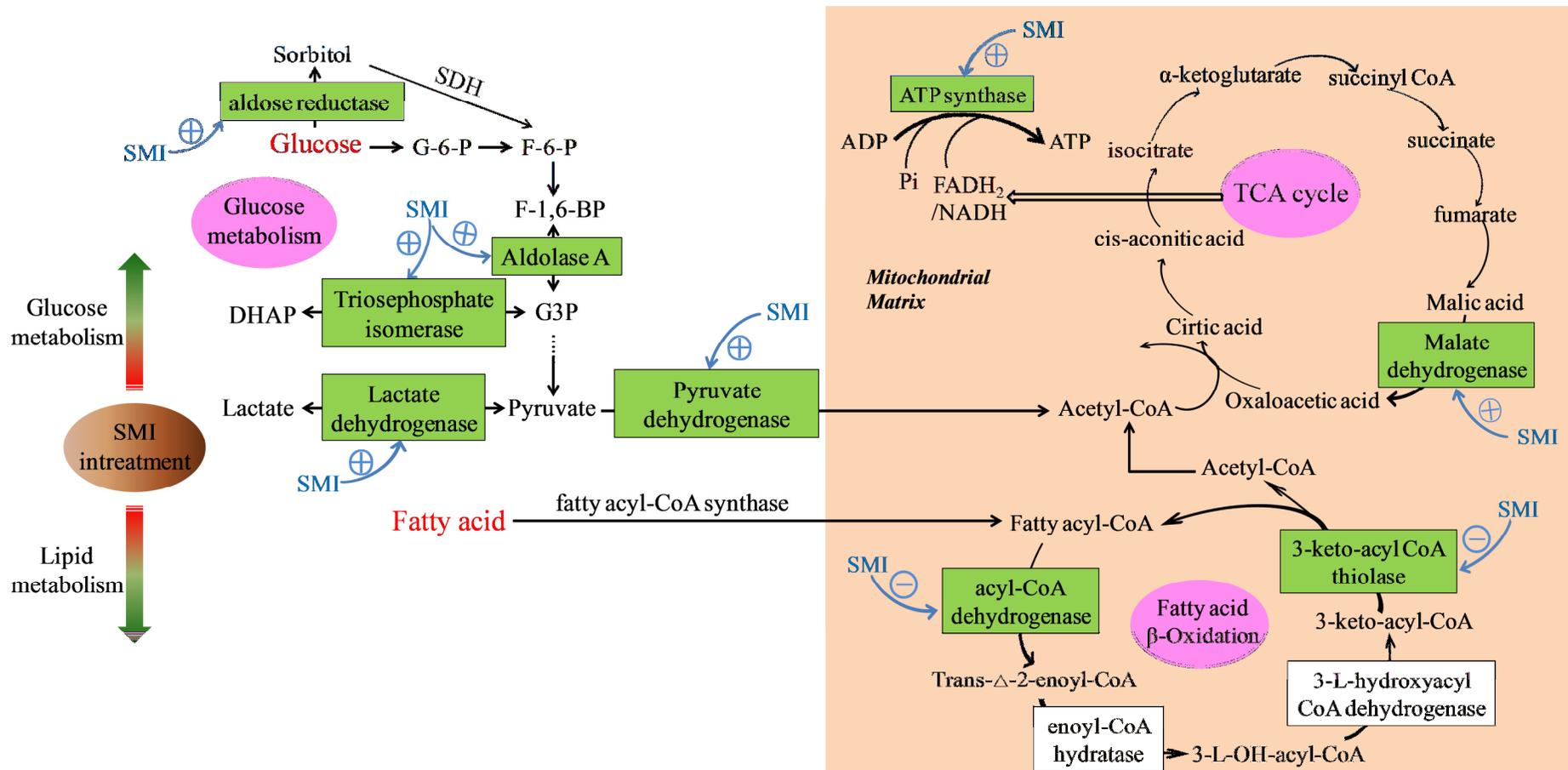


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**Fig. 6.** Modulation of myocardial energy metabolism by SMI treatment; G-6-P, Glucose-6-phosphate; F-6-P, Fructose-6-phosphate; F-1,6-BP, Fructose-1,6-bisphosphate; G3P, Glyceraldehyde-3-phosphate; SDH, Sorbitol dehydrogenase; DHAP, Dihydroxyacetone phosphate; TCA, Tricarboxylic acid