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RESEARCH ARTICLE

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Different protein expression of myocardium from Chinese mini-swine model of myocardial infarct

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Abstract High-resolution two-dimensional gel electrophoresis (2-DE), followed by computer-assisted image analysis was used to screen protein patterns of normal and infarcted myocardial tissues for quantitative and qualitative differences in protein expression. In the gels of pH 5–8 immobilized pH gradient (IPG) strips, 851 protein spots were detected in normal myocardial tissue and 1 032 protein spots were resolved in infarcted myocardial tissue. Thirteen protein spots only expressed in normal myocardial tissue, and 14 protein spots only expressed in infarcted myocardial tissue. Results also showed that 49 protein spots displayed quantitative changes in expression between normal and infarcted myocardial tissue. Eleven protein spots were subjected to mass spectrometry (MS) analysis and seven proteins were identified by peptide mass fingerprinting (PMF). These proteins may be involved in cardiovascular injury, and could play an important role in the treatment of coronary heart disease.

Keywords myocardial infarct, myocardial tissue, proteome, two-dimensional gel electrophoresis

1 Introduction

Recently, proteomics has been widely used to investigate the protein patterns of different cardiovascular diseases, such as heart failure [1], dilated cardiomyopathy (DCM) [2], arteriosclerosis [3], and myocardial infarction of different animal models, such as dog [4], rabbit [5], and rat [6,7], and some identified proteins are demonstrated as having a correlation with the onset of diseases [8,9].

By cardiac catheterization, an embolus was transplanted into the coronary arteries of a Chinese mini-swine, which is similar to those of human beings. The embolus blocked the coronary blood flow and caused myocardial infarction. Thus, a myocardial infarct model, which can be used in both acute and chronic experiments, was established [10]. This is a pathologic and physiological course similar to a clinical condition. The goal of the present study was to identify the protein changes in cardiac protein expression after myocardial infarction. Techniques in proteomic approach were used to investigate the protein expression profiles between normal and infarcted myocardial tissues. Using 2-DE (two-dimensional gel electrophoresis), MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) and PMF (peptide mass fingerprinting), we identified seven proteins significantly altered during the process of myocardial infarction. These altered proteins may play important roles in the onset of myocardial infarction.

2 Materials and methods

2.1 Animal model and preparation of tissue samples

Porcine were anesthetized by an injection of sodium pentobarbital (20 mg/kg). By cardiac catheterization, an embolus was transplanted into the coronary arteries of Chinese mini-swine according to the protocol of Mortz et al. [11]. The embolus blocked the coronary blood flow and caused myocardial infarction. Thus, a myocardial infarct model, which can be used in both acute and chronic experiments, was established. After two weeks, the heart was rapidly removed after killing the mini-swine. Normal and infarcted myocardial tissue samples were cut from the heart, separated and stored at –80°C, respectively.

Tissue samples were cut into small pieces and then homogenized on ice in a lysis buffer containing 9 M urea (Promega), 4% CHAPS (Promega), 0.5% DTT (Promega) and 0.14% PMSF (Promega). The homogenate was collected into microcentrifuge tubes and sonicated in ice-water for 10 min with the addition of DNase-RNase solution(Roche). After being stored at 4°C for 15 min, samples were centrifuged at 15 000 r/min for 30 min at 4°C and supernatants

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were collected. Total protein concentration was measured by use of a Bradford Assay.

2.2 Two-dimensional gel electrophoresis and protein visualization

Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) strips (pH 5−8, 17 cm, linear gradient, Bio-Rad) on a Protean cell (Bio-Rad). The samples were diluted with rehydration solution containing 8 M urea, 4% CHAPS, 65 mM DTT, 0.2% ampholyte and 0.001% bromophenol blue, and 400 μL of each sample (100 μg of protein) was subjected to an IPG strip and active rehydration was performed for 15 h at 50 V. Proteins were focused in a four-phase program: (i) step-and-hold 250 V for 30 min; (ii) gradient 1 000 V for 1 h; (iii) gradient 10000 V for 5 h; (iv) step-and-hold 10 000 V, were performed until 60 000 Vh. After IEF, strips were equilibrated for 10 min in the equilibration buffer (6 M urea, 375 mM Tris-HCl, 20% glycerol, 2% SDS and 2% DTT), followed by the same equilibration buffer with 2.5% iodoacetamide (Bio-rad) instead of 2% DTT for another 10 min. SDS-PAGE was performed using 12% polyacrylamide gels on a Protean II XL system (Bio-Rad). Electrophoresis was carried out at 10 mA per gel for 30 min, followed by 30 mA per gel for about 8 h; the temperature of the electrophoresis system was kept at a range of 4°C−5°C. Protein spots on the analytical gels were detected with silver-staining according to the protocol described by Yan et al. [12].

2.3 Image analysis

2-DE gel images were acquired using a PowerLook 2100 XL scanner system (UMAX Data Systems, Inc). Protein spots were then located and quantified with PDQuest 7.1 software (Bio-rad). The quantity of each spot was normalized by total quantity in valid spots using the software. This normalized value was used to calculate the mean quantity of each spot per group. A statistical comparison of the relative abundance of each matched protein spot between normal tissues and infarcted tissues was accomplished.

2.4 In-gel tryptic digestion and mass spectrometry analysis

From the 2-DE gel analysis of normal and infarcted myocardial tissue samples, differentially expressed proteins were selected for identification by mass spectrometric analysis. Protein spots were excised from 2-DE gels, cut into 1 mm cubes, and then destained according to Gharahdaghi et al. [13]. Protein samples digested by trypsin were analyzed using a time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex). Peptide matching was carried out against the NCBI nonredundant databases and Swiss-Prot database using the Mascot, Profound search software.

3 Results and discussion

3.1 Effect of IPG strips with different range

For the purpose of initially scanning the protein expression of normal myocardium of Chinese mini-swine, we used IPG strips with a wide range, linear IPG (pH 3−10) in the first dimension. About 830 proteins were detected by the PDQuest software. In the wide-range 2-DE maps, there was a loss of resolution in the region pH 5−8, which was most probably due to the fact that the *p*I values of many proteins occurred in this range. Therefore, we also performed electrophoresis on pH 5−8 and pH 4−7 to achieve a better protein separation. Figure 2 showed the same part of protein expression between the 2-DE maps using IPG strips with wide (pH 3−10) and narrow (pH 4−7) pH ranges. These narrow pH gels allowed a higher resolution and more protein spots in the relative pH zones. Therefore, we used IPG strips with narrow pH range during the latter experiment.

3.2 Reproducibility of the protein expression in 2-DE maps

We explored protein expression of normal tissue samples using linear IPG strips (pH 4−7) under the same condition for four times. Twenty-one clear and matched protein points were selected, and one of them was set as the reference point.

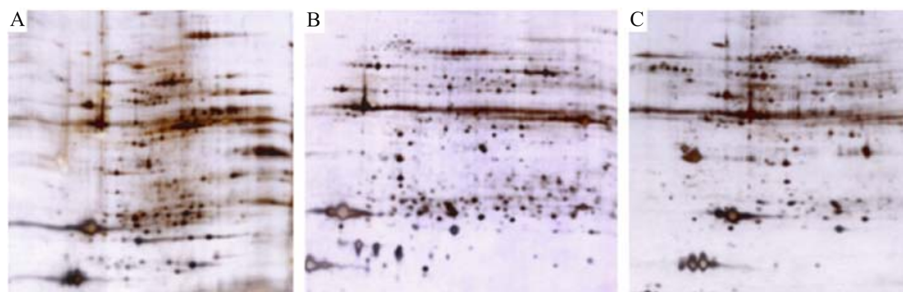

Fig. 1 2-DE profiles of the protein expression of normal myocardial tissues
Proteins were detected by silver staining. The 17 cm-IPG strips (pH = 3−10) were used in (A), strips (pH = 5−8) were used in (B) and strips (pH = 4−7) were used in (C)

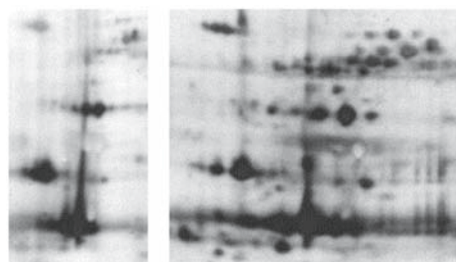

Fig. 2 Comparison the resolution of 2-DE maps with IPG strips (pH = 3−10) (left) and strips (pH = 4−7) (right)

The distance from the reference point to other protein points were measured in rectangular coordinates in two dimensions (X-coordinates stands for IEF; Y-coordinates stands for SDS-PAGE). The standard deviations (Sx, Sy) of the distance in the four 2-DE maps at both X and Y directions were calculated to test the precision of the protein expression in 2-DE maps. The distances were displayed from 0.6 to 100.6 mm, and the standard deviations were from 0.3 to 2.5 mm. Therefore we could get the inclusion that 2-DE was a precise and steady technology. The average of Sy was 1.38 mm which was larger than the average of Sx at 1.23 mm, and it meant that the precision during IEF was much better than that during SDS-PAGE.

3.3 Comparison of protein patterns between normal and infarcted myocardial tissues

The profile patterns of proteins from normal and infarcted myocardial tissues were obtained by 2-DE with pH 5−8 IPG (as Fig. 3 showed). Following analysis of the protein patterns using PDQuest 7.1 software, on an average of 851 protein spots was detected in the patterns of normal tissues and 1 032 spots were found in the patterns of infarcted tissues. When the percentage volume of the matched spots was analyzed, it was clear that the expressions of nearly half of the protein spots remained unaltered. However, 13 protein spots only expressed in normal myocardial tissue (as Fig. 3(A) A01−A13 showed), and 14 protein spots only expressed in infarcted myocardial tissue (as Fig. 3(B) B01−B14 showed). There was a group of 49 protein spots that was significantly altered between normal and infarcted myocardial tissues. Of the group, 18 protein spots (as Fig. 3(B) C01−C18 showed) were up-regulated in the protein pattern of infarcted tissues and 31 protein spots (as Fig. 3(B) D01−D31 showed) were down-regulated. These results illustrated that there were marked changes in protein expression during the onset of myocardial infarction in mini-swine.

3.4 Protein identification

Based on the comparison of protein profiles of normal and infarcted myocardial tissues, 11 protein spots that differently expressed were subjected to mass spectrometry for protein identification and seven altered proteins were identified according to the Mascot search engineering. The summary of identified proteins is shown in Table 1.

Protein identities were confirmed by Mascot and Profound by using the MS/MS peak lists exported from mass spectra data. The nonredundant data bases in the molecular mass range of 1 000−500 000 Da and pI values between 3.0 and 10.0 were used at the NCBI and Swiss-Prot website. Modifications considered included carbamidomethylation of cysteine, amino-terminal acetylation, amino-terminal Gln to pyro-Glu, oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine [14]. The molecular weights and pI values of B05, B06, C02, C03 and C10 represented

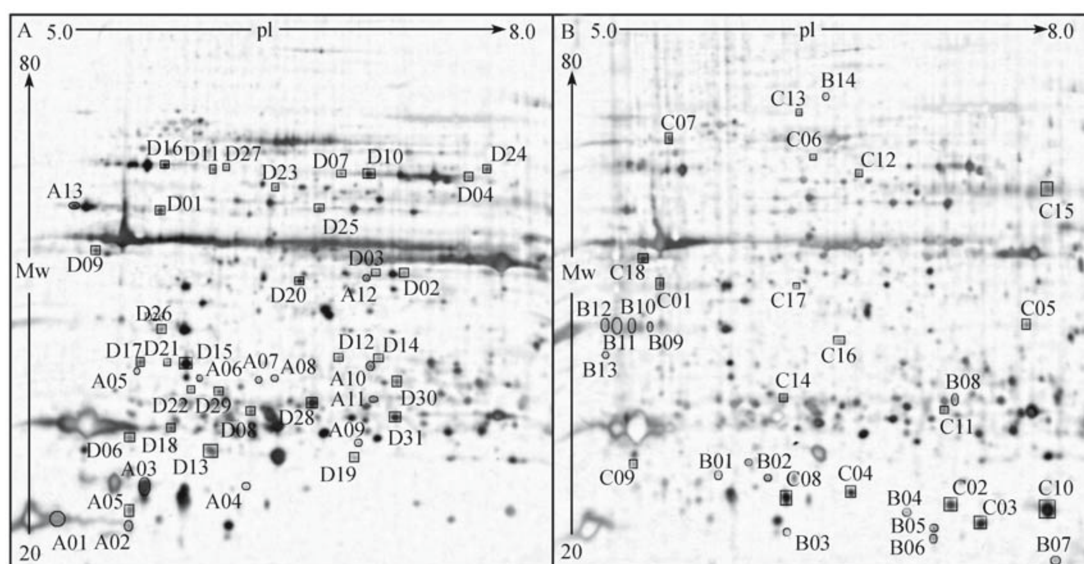

Fig. 3 The proteins expressed differently between (A) normal myocardium and (B) infracted myocardium

Table 1 Proteins identified by MS

NO	Protein name (gene name)	AC for databases	MW/pI (theoretical)	Score	Matched peptides /all peptides	Seq. cov. /%
A11	Tubulin beta chain	gi|135 490 P02 554	32.1/6.53 (49.83/4.78)	161	18/29	41
B05	Alpha-b crystallin (Cryab)	gi|7 441 290 P41 316	20.5/6.67 (20.12/6.76)	64	6/35	44
B06	Alpha-b crystallin (Cryab)	gi|7 441 290 P41 316	20.0/6.67 (20.12/6.76)	66	7/43	46
C02	Alpha-b crystallin (Cryab)	gi|7 441 290 P41 316	21.9/6.75 (20.12/6.76)	102	11/61	53
C03	Alpha-b crystallin (Cryab)	gi|7 441 290 P41 316	20.8/7.05 (20.12/6.76)	118	12/54	62
C10	Alpha-b crystallin (Cryab)	gi|7 441 290 P41 316	21.0/7.15 (20.12/6.76)	161	17/62	66
C08	Myosin heavy chain, cardiac muscle beta isoform (fragment) (MYH7)	gi|55 741 486 P79 293	22.3/5.91 (223.66/5.59)	327	45/50	28
D07	Microsomal protease ER-60	gi|1 583 929	55.5/6.36 (57.01/5.88)	224	20/34	42
D10	Lipoamide dehydrogenase	gi|47 522 940 P09 623	56.6/6.52 (54.72/7.59)	177	17/34	45
D25	Alpha enolase (Eno1)	gi|4 927 286 Q9XSJ4	51.0/6.29 (47.59/6.44)	89	10/28	24
D26	Cardiac alpha tropomyosin (TPM1)	gi|1 927 P42 639	35.8/5.53 (32.72/4.69)	137	18/56	51

differently, but they were the same protein with different modifications which was confirmed by Mascot and Profound. Taking protein spot C03 as an example (Fig. 4), the sequence coverage of which was 58%, an amino group (APSWIDTGLSEMR) was oxidated; two amino groups (APSWIDTGLSEMR and APSWIDTGLSEMRLEK) of protein spot C10, whose sequence coverage was 72%, were oxidated. That was why the same protein expressed differently in 2-DE maps.

Amino acid sequence of alpha-b crystalline was shown down, and matched amino groups of C03 and C10 were marked with grey.

C03:
1 MDIAIHHPWI RRPFFPFHSP SRLFDQFFGE HLLESDLFPA STSLSPFYFR
51 PPSFLRAPSW IDTGLSEMRL EKDRFSVNLD VKHFSPEELK VKVLGDVIEV
101 HGKHEERQDE HGFISREFHR KYRIPADVDP LTITSSLSSD GVLTVNGPRR
151 QASGPERTIP ITREEKPAVT AAPKK

C10:
1 MDIAIHHPWI RRPFFPFHSP SRLFDQFFGE HLLESDLFPA STSLSPFYFR
51 PPSFLRAPSW IDTGLSEMRL EKDRFSVNLD VKHFSPEELK VKVLGDVIEV
101 HGKHEERQDE HGFISREFHR KYRIPADVDP LTITSSLSSD GVLTVNGPRR
151 QASGPERTIP ITREEKPAVT AAPKK

3.5 Functional analysis of the identified proteins

After protein identification, we analyzed the information of these identified proteins from the Swiss-Prot and NCBI databases. Some interesting proteins, such as tubulin, alpha-B crystalline, myosin, tropomyosin and lipoamide dehydrogenase, were regulated with myocardial infarct.

Microtubules play an important role in maintaining cellular structural integrity. In intact myocardium, microtubules were stained as a filamentous network throughout the cytoplasm and a circular network around the nucleus. Iwai et al.'s research [15] pointed out that with an increase in ischemic period, lesions of loss of microtubule stains were increased in number and size and that microtubules that support the structural integration of myofibrils and other organelles are disrupted in severe myocardial ischemia before the irreversible injury, promoting the irreversible change after reperfusion.

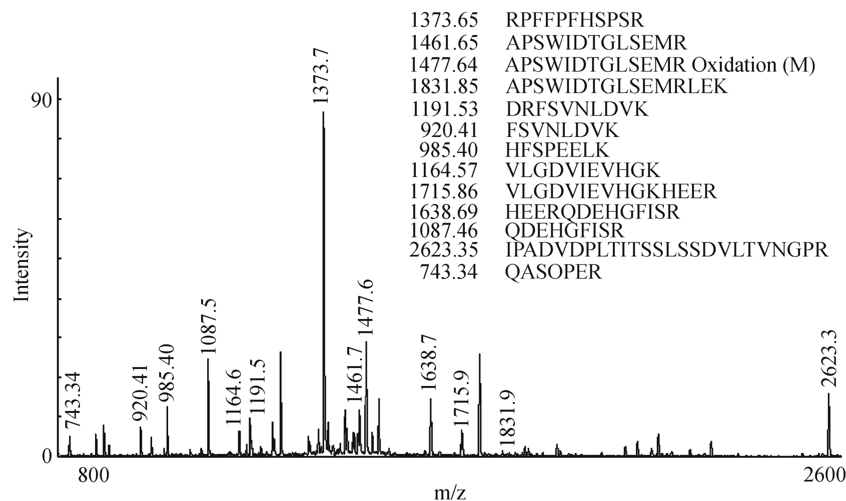

Fig. 4 MS spectrum of protein spot C03

The sarcomere is the basic contractile unit of the heart, and is comprised of both thick and thin filaments. The main component of the thick filament is the myosin heavy chain, while the thin filament is composed of actin, α-tropomyosin and the troponins I, C and T. Myosin-binding protein C and titin are major components of the sarcomere and are involved in both the stabilization of the sarcomere structure, and the generation and transmission of force. Following activation by Ca^{2+}, a series of events, involving the troponin-tropomyosin complex, results in the sliding of the thin and thick filaments and consequently in sarcomere shortening and muscle contraction. One of the molecular biology characteristics of myocardial infarct is the decrease of contractile proteins.

In the human adult heart, the predominant tropomyosin isoform (α-Tm), stabilizes and stiffens the filament and, in the absence of calcium, blocks the myosin-binding site of actin. It may also play a role in determining the degree of cooperativity and calcium sensitivity [16].

There is a great deal of evidence that the rate of rise of force in myocardium varies with the expression of myosin heavy and light chain isoforms, which in turn varies between species, within species depending on developmental stage, and in diseases such as heart failure [17]. The cooperative activation of thin filaments has significant implications for myocardial function. Cooperativity in crossbridge binding and thin filament activation is likely to influence or mediate other functional characteristics of myocardium, and derangements in cooperative processes may underlie contractile dysfunction in some myocardial diseases. There is growing evidence that the strong binding of myosin crossbridges to the thin filament cooperatively accelerates the rate of force development in both cardiac and skeletal muscles [18].

HSP27 and alpha-B-crystallin are a group of stress related proteins in response to heat and other environmental stress. The small heat shock protein alpha-B crystalline is a molecular chaperone whose function is to stabilize proteins damaged by inflammatory stress. It has been shown that alpha-B crystalline and Hsp27 translocate to the myofilament/cytoskeletal compartment during ischemic preconditioning. Additional phosphorylation of alpha-B crystalline on Ser-59 seems to be indispensable for the early protection after ischemic preconditioning [19]. In our experiment alpha-B crystalline showed up-regulation observably in infracted myocardium tissue.

Lipoamide dehydrogenase and alpha enolase are enzymes related with energy metabolism. Lipoamide dehydrogenase, a component of the pyruvate, alpha-ketoglutarate, and branched chain alpha-ketoacid dehydrogenase complexes, is an important enzyme that catalyzes the conversion of pyruvate to acetyl-CoA for metabolism through the tricarboxylic acid cycle. The corresponding gene of lipoamide dehydrogenase was down-regulated observably after ischemic preconditioning in rats as Lv et al.'s research [20], which was consistent with our result. Alpha enolase is a multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. It may also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. Recently, it is reported that the mRNA and protein expression of enolase were down-regulated after ischemic preconditioning to protect the myocardium.

4 Conclusions

Two-dimensional electrophoresis acts like a molecular microscope. Complex protein mixtures are separated into distinct protein species. Comparing diseased with normal ones elucidate disease-associated proteins. In conclusion, it is expected to be a useful tool for elucidating proteins associated with heart failure and heart disease. This study described a proteomics approach to investigate the protein patterns between normal and infarcted tissues from a mini-swine myocardial infarction model. These identified proteins could be induced or resisted under pathological conditions, suggesting that this phenomenon may be exploited for therapeutic purposes.

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