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RESEARCH ARTICLE

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Proteome analysis of differential protein expression in infarcted rat heart after verapamil treatment

Abstract To explore the protein-level mechanism of action verapamil in acute myocardial infarcted rats, the myocardial proteome was analyzed by two-dimensional electrophoresis (2-DE). Compared with the sham-operated group and the infarcted group, the result shows that 8 protein expressions in the verapamil treated group were up-regulated, and 7 protein expressions in this group were down-regulated significantly. Using MALDI-TOF-MS, 15 proteins with significant changes were identified through a database search. These proteins can be divided into 4 groups by their biological function: (1) Energy metabolism and mitochondrial function related proteins; (2) oxidative stress-induced proteins; (3) cytoskeletal Proteins; (4) other proteins. The findings show that the myocardial protective effects of verapamil in the myocardial damage process are related to the recovery of energy supply as well as anti-oxidative stress property.

Keywords verapamil, proteomics, two-dimensional electrophoresis (2-DE), myocardial ischemia

As a mature calcium channel blocker, verapamil has been widely used in the clinical treatment of arrhythmia, hypertension and other cardiovascular diseases [1, 2]. Through inhibiting extracellular calcium ions uptake through the cell membrane and reducing free calcium concentration in vascular smooth muscle cells, verapamil causes vasodilatation of the coronary and peripheral vessels, reduces cardiac autorhythmicity and the conduction velocity of atrioventricular and sinoatrial node to produce its effects. Although a considerable amount of researches have explained the action mechanism of verapamil from different views [3], clinical studies have also shown that verapamil can reduce the re-infarction and mortality rates of patients (without heart failure after acute myocardial infarction) [4]. However, differential protein expression regulated by verapamil after acute myocardial ischemia has not been reported yet.

Proteomics technology has not only applied for studying the pathophysiology process of various diseases and discovering possible drug targets [5, 6], but has also elaborated drug action models, side effects, toxicity and drug resistance mechanisms [7]. As the most extensively applied separation method in proteome analysis, two-dimensional electrophoresis (2-DE) has been used for analyzing the changes of differential proteins of tissue and cell samples [8, 9]. In this paper, we used comparative proteomic method to study the altered proteins of rat myocardial tissues after acute myocardial infarction with verapamil treatment, and investigate the possible anti-myocardial ischemic mechanism of verapamil at the protein level.

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1 Materials and methods

1.1 Reagents, equipment and animals

HCl-verapamil (lot number: 6E12004) was purchased from the Shanghai Hefeng Pharmaceutical limited company (Shanghai, China). Bisacrylamide(bis), Tris(hydroxymethyl) aminomethane (Tris), Sodium dodecyl sulfate (SDS), Glycine, N,N,NU,NU-tetramethylethyldiamide (TEMED), Ammonium persulfate (APS), Glycerol, ultra pure Urea, 2-D cleanup kit, 2-D Quant Kit, IPG-strips (24 cm, pH 3-10) and IPG buffer were purchased from GE Healthcare (Amersham, Freiburg, Germany). The Lactate dehydrogenase (LDH), Creatine kinase (CK) and CK-isoenzyme (CK-MB) kits were purchased from Nanjing Jiancheng Technology Co. Ltd. (Nanjing, China). Acrylamide, Dithiothreitol (DTT), 3-3-1-propane-sulfonate (CHAPS), Coomassie G-250 (ultra pure grade) and Agarose were purchased from the Shanghai Biotech Co. Ltd (Shanghai, China). Iodoacetamide (IAA) was pur-

chased from Fluka BioChemika (Buchs, Switzerland). HPLC-grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). HPLC-grade trifluoroacetic acid (TFA) was purchased from Tedia (Fairfield, USA). Formic acid (FA) was purchased from Acros Organics (Fairlawn, NJ). All other solvents were of analytical grade.

The 2-DE apparatus, which contains Ettan IPGphor and Ettan DALTsix, were purchased from GE Health (Amersham, Freiburg, Germany). The Umax PowerLook 2100XL Scanner was purchased from Umax (Umax, Hanchu, Taiwan). Image analysis software ImageMaster 2D Platinum was obtained from Amersham (Amersham Biosciences, USA). Mass spectrogram analyses were performed on 4700 Proteomics Analyzer (TOF/TOF™) (Applied Biosystems, USA).

Male Sprague-Dawley rats were purchased from the Zhejiang Experimental Animal Center. All rats were provided with water and chow *ad libitum*. The animal usage conforms to the Guide for Care and Use of Laboratory Animals of the Zhengjiang University.

1.2 Myocardial infarction, treatment and histological study

24 male Rats were randomly divided into three groups: the sham group ($n = 8$), myocardial infarction (MI) group ($n = 8$), verapamil treatment (MI + verapamil) group ($n = 8$). 4 hearts of each group were examined to estimate infarct size after heart collections, and the other hearts were used to proteomics analysis. Myocardial infarction was induced by ligating the left anterior descending coronary artery according to the method of Yamaguchi [10]. The sham group and MI group rats were given normal sodium (*i.p*), while MI + verapamil group rats were given verapamil (1 g/kg). All experimental rats were sacrificed 24 h after treatments by being bled from inferior vena cava. The hearts were quickly removed and perfused with oxygen-saturated Krebs-Henseleit solution for 1 min. Then, rapid removal of fat and connective tissues was done, and then the hearts were cut at the ligation area into 2 pieces. The tissues were frozen under ligation (without right ventricle) in liquid nitrogen and stored at −80°C.

The heart slices were incubated in 1% triphenyltetrazolium chloride (TTC), and corresponding areas were weighed out. The weight percentage of infracted area in total heart was considered to evaluate drug actions. Serum contents of LDH, CK, CK-MB were determined by the use of diagnostic kit according to the supplier's instructions.

1.3 Protein extraction and 2-DE analysis

Protein extraction and 2-DE analysis were according to the method of reference [11]. IPG-Strips (24 cm non-linear gradient, pH 3–10) with proteins were rehydrated for 12 hours at 30 V, then focus for 1 hour at 100 V, 1 hour at 200 V, 1 hour at 500 V, 1 h at 1000 V, 30 min for voltage increasing to 8000 V, and remain 8000 V for 73 kVh on the IPGPhor. The equilibrated IPG-strips were applied onto a 12.5% polyacrylamide gradient gel (26×20 cm). First separation was performed sequentially with a constant voltage of 5 W/gel for 1 hour, followed by 20 W/gel for 6 hours using the Ettan DALT system. After SDS-PAGE, the gels were visualized by Coomassie Brilliant Blue (CBB) or silver staining.

1.4 Gel image analysis and protein identification

Silver-stained gels were scanned using the Powerlook 2100XL scanner and the gel images were analyzed using ImageMaster 2D Platinum software (analysis parameters: smoothing degree is 2, minimum area is 5). The intensity of each spot was expressed with the percentage of optical density values. The percentage of interclass protein volumes was considered to be a standard to define disparate points, whose optical density difference was more than 2-fold. A subset of the most promising candidate spots were selected for preparation for MS.

CBB-stained gels were used for harvesting proteins for identification. After cutting altered protein spots, the decolorization and in-gel digestion of CBB-stained spots, and the detection of peptide mass fingerprinting (PMF) were performed according to the method by Jensen [12]. Using the program MASCOT (Matrix Science, London, UK) against an NCBInr database, each search was limited in *rattus norvegicus* species. The maximum of noise tolerances were PMF tolerance of 0.3 Da, and MS/MS tolerance of 0.4 Da.

2 Results and discussion

2.1 Effect of verapamil on infarct size and serum CK and CK-MB level

The contents of myocardial infarction area, serum creatine kinase (CK) and its isoenzyme-MB (CK-MB), as well as the important physiological and biochemical indicators, were used to evaluate the cardioprotective effects of verapamil against acute ischemic myocardial. There is little myocardial infarction area in the sham group, and 23.1%± 4.42 in the MI group (IA/H). Treatment with verapamil resulted in a smaller infarction with 12.6%±3.67 ($P < 0.01$, Table 1).

Table 1 Alteration of infarcted size, LDH, CK and CK-MB in different groups

	Iinfarcted size/%	LDH (U/L)	CK (U/L)	CK-MB (U/L)
MI	23.1±4.42	1646±95.7	706±59	1012±106.4
Sham	0.4±0.24**	700±209.3**	329±41.1**	539±58.5**
MI + verapamil	12.6±3.67**	944±143.3**	516±84.4*	777±121.4*

Compared to MI group, *$P < 0.05$, **$P < 0.01$

Serum CK and CK-MB, important cardiac-specific markers of acute myocardial infarction, indicate degrees of myocardial injury. Treatment with verapamil resulted in a marked reduction in serum CK and CK-MB activity, compared with the MI group rats (516±84.4 and 777±121.4 U/L versus 706±5.9 and 1012±106.4 U/L, $P < 0.05$ and $P < 0.01$, respectively). Meanwhile, CK and CK-MB in sham group rats were 329±41.1 and 539± 58.5 U/L (Table 1). These results indicated that verapamil could effectively improve the cardioprotective effects against myocardial infarction induced by acute ischemic myocardial, and it was consistent with those reports of clinical effects of verapamil treatments in myocardial infarction, angina and coronary heart disease [13].

2.2 Results of proteomic analysis

2.2.1 Differential expression of rat heart proteins

Protein samples from the Sham, MI and MI + verapamil groups were prepared as described above. Then, the samples were analyzed with 2-DE (repeated three times each sample). The spots were visualized by silver staining (shown in Fig. 1). Using the Image Master 2D Platinum, the means of the 750, 760 and 820 protein spots were detected from the Sham, MI and MI + verapamil group, respectively. The standard deviation of protein point numbers and optical density value in reduplicate gels were less than ±10% and ±15%. As can be seen from Fig. 1, most of the protein spots were distributed in the region of isoelectric point (p*I*) 4–9 and had molecular weight between 15–70 kDa. Compared with MI group, 15 protein spots were found to be significantly altered in MI + verapamil group. Among these proteins, 8 proteins were up-regulated, and 7 proteins were down-regulated.

2.2.2 Identification of altered proteins

Considering the sham group protein expression as a standard, the altered protein spots of MI and MI + verapamil group were identified using MALDI-TOF-MS. The relative abundance and identification results are listed in Table 2.

The altered proteins identified by MS were categorized into four groups according to their functions. The first group was associated with energy metabolism. The up-regulated ones includes the mitochondrial precursors of NADH ubiquinone oxidoreductase, NADH dehydrogenase 1 alpha 10 (NDUFA10), ATP synthase, malate dehydrogenase 1, long-chain acyl-CoA dehydrogenase, aldolase A and creatine kinase. The down-regulated ones includes lactate dehydrogenase B and enolase 3-*β*. The second group was related to oxidative stress, including the increase in 58 kDa glucose regulated protein (Grp58) and the decrease in *α*B-crystallin and heat shock protein 27 (Hsp27). The third group was associated with cytoskeleton, including myosin polypeptide 6 and light polypeptide 3. Other proteins, like the Annexin III, was increased.

As a commonly used calcium antagonist, verapamil can expand the main coronary artery and small arteries somewhat at the normal position or non-ischemic state of heart, by increasing the oxygen and other nutrients delivery via the coronary circulation and offset vascular tone to ease the influence of myocardial ischemia. The Data in Section 2.1 show that verapamil exerted significant cardioprotective effects against acute ischemic myocardial injury in rats, and proteomics research findings provided explanations on the molecular level.

About a half of the identified proteins were involved in the energy metabolism and mitochondrion function. β-oxidation and tricarboxylic acid cycle play important roles in energizing for myocardium. After ligating the left anterior descending coronary artery, the expression of long chain acetylcoenzyme A and aldolase A were significantly decreased, suggesting β-oxidation and tricarboxylic acid cycle were suppressed enormously with prolonged deficiency of oxygen and other substrates. However, treatment with verapamil could reverse the effect of suppression, suggesting an improvement of energy provision. NADH

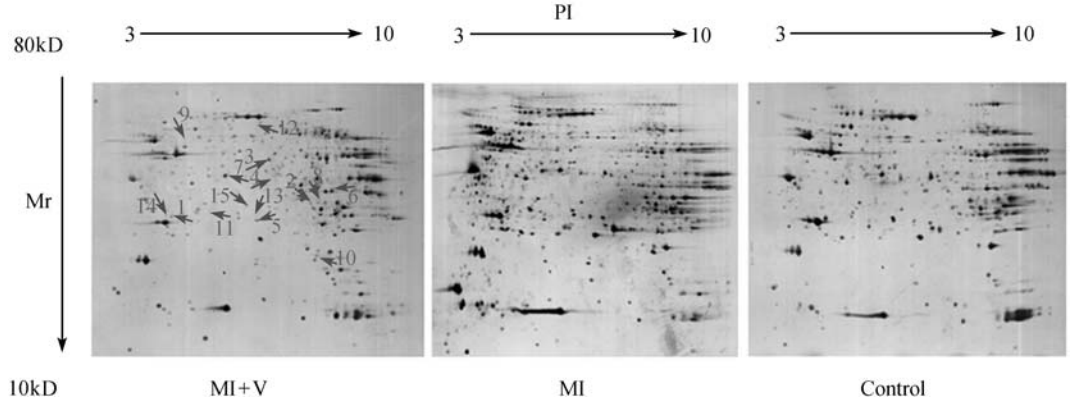

Fig. 1 Representative 2-D gel images for verapamil treated (MI + V), infracted (MI) and sham-operated (Control) group

Table 2 Differentially expressed proteins in Verapamil treated group*

	Protein identity	Function	NCBI accession No.	p*I*	Mr/kDa	Score	Expression compared to control	
							MI	MI + V
	NADH ubiquinone oxidoreductase (24-kDa subunit)	mitochondrial function	83305118	4.8	22	126	↓	↑
	ATP synthase, H(+)-transporting	mitochondrial function	57029	7.0	25	118	↓	↑
	NADH dehydrogenase 1 subunit alpha 10	mitochondrial function	32996721	6.3	41	206	↓	↑
	malate dehydrogenase 1	energy metabolism (*TCA-cycle*)	15100179	6.3	36	199	↓	↑
	long-chain acyl-CoA dehydrogenase	energy metabolism (*β-oxidation*)	6978431	6.3	21	145	↓	↑
	aldolase A	energy metabolism (*glycolysis*)	202837	7.6	37	99	↑	↑
	lactate dehydrogenase B	energy metabolism (*glycolysis*)	6981146	5.7	37	181	↓	↓
	creatine kinase, muscle	energy Metabolism (*Phosphocreatine*)	6978661	7.0	43	110	↑	↑
	enolase 3, beta	energy metabolism	126723393	7.08	47	203	↑	↓
0	alpha B-crystallin	heat shock protein	57580	6.8	19	178	↑	↓
1	heat shock protein 27	heat shock protein	14010865	5.6	21	119	↑	↓
2	glucose regulated protein, 58 kDa	stress-induced	38382858	6.2	60	293	↓	↑
3	myosin heavy chain, polypeptide 6	cytoskeletal protein	554475	6.2	20	80	↓	↓
4	myosin, light polypeptide 3	cytoskeletal protein	6981240	4.8	20	137	↑	↓
5	annexin A3	other Protein	122065130	5.9	36	135	↓	↓

*↑: up-regulated; ↓: down-regulated.

ubiquinone oxidoreductase is an important component in the mitochondrial electron transfer chain complexes. In anaerobic conditions, the velocity of ATP and ADP transfer through the mitochondrial inner membrane and the oxidative phosphorylation of cytochrome oxidase were both influenced [14], and the expression of NADH ubiquinone oxidoreductase was decreased by acute myocardial ischemia. Compared with the MI group, verapamil treatment could prevent these decreases. In addition, the expression of proteins associated with glycolysis and phosphocreatine was increased in the MI + verapamil group, suggesting verapamil can partially promote glycolysis and phosphorylation of sarcosine to energize to ischemia tissues.

Myocardial ischemia also leads to differential expression of proteins associated with stress response. Heat shock protein (HSP) and other proteins participate in cellular self-adapting protection during the process of ischemia [15]. In our study, two altered HSPs were identified as alpha B-crystallin and Hsp27. The expression was significantly increased in the MI group, but decreased with verapamil treatment, demonstrating that oxidative stress can be partially suppressed with verapamil treatment.

Myosin, one of the cytoskeletal proteins, takes responsibility for contraction. In our study, the expression alternations of skeletal proteins in ischemic heart tissues was consistent with the previous proteomics study [16]. In addition, Annexin A3, associated with calcium ion binding in cell membrane, generated a significant change after verapamil treatment, and its mechanism is worthy of further study.

In summary, verapamil can exert conspicuous cardioprotective effects, through the production of significant deviation of protein expression, which leads to partial recovery of myocardial energy metabolism and mitochondrial function, and down-regulation of the expression of oxidative stress proteins.

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