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Resveratrol Protects Left Ventricle by Increasing Adenylate Kinase 1 and Isocitrate Dehydrogenase Activities in Rats with Myocardial Infarction

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Abstract

Our prior study had shown that resveratrol was a potent cardioprotective agent in rats with myocardial infarction (MI). In this study, we further evaluated the mechanism of cardioprotection of resveratrol by proteomic analysis. After permanent ligation of the left anterior descending artery under isoflurane anesthesia, surviving rats were randomly allocated to three groups and treated with resveratrol at 1 mg/kg/day (MI/R group), or vehicles (sham group and MI group) once daily for four weeks. In proteomic analysis, the MI group showed decreased expression of adenylate kinase 1 (AK1) and mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) after MI compared with the sham group. These variations were reversed by resveratrol in the MI/R group. Validation with Western blot and immunohistochemical analyses showed similar trends in protein expression profiling. Our studies suggest that the beneficial effects of resveratrol on ventricular modeling may be due to increased expression of AK1 and IDPm, which have been known to increase myocardial energetic efficiency and reduce reactive oxygen species-mediated damage, respectively.

Key Words: resveratrol, adenylate kinase 1, isocitrate dehydrogenase, myocardial infarction

Introduction

Myocardial infarction (MI) is most commonly caused by coronary artery occlusion. After prolonged ischemia, myocardial necrosis occurs and leads to left ventricular (LV) remodeling. LV remodeling is char-

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acterized by progressive LV dilatation and systolic dysfunction associated with an increased risk of congestive heart failure (20). Despite reduction of the mortality rate of MI by treatment with β blockers (5) and angiotensin-converting enzyme inhibitors (19), the incidence of heart failure increases in MI survivors (25). Therefore, the pathophysiology of heart failure after MI needs more investigation.

Adenylate kinase is a phosphotransferase enzyme that catalyzes the interconversion of ATP and ADP and is able to provide ATP rapidly during intense activity. The reaction catalyzed is: 2 ADP → ATP + AMP (28). In myocardial and vascular tissues, adenylate kinase 1 (AK1) is the major adenylate kinase isoform. AK1 gene deletion lowers myocardial energetic efficiency, compromises the contractile force, reduces tolerance to ischemic stress and decreases coronary reflow following ischemia-reperfusion (6, 21). However, the biological importance of AK1 in MI has not been completely established.

In eukaryotes, isocitrate dehydrogenase exists in two forms: an NAD^+-linked enzyme found only in mitochondria and an $NADP^+$-linked enzyme found in both mitochondria and cytoplasm (15). Mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) plays a significant role in defending against oxidative damage by providing NADPH (27). Decreased expression of IDPm markedly increases cell death rate, DNA fragmentation, intracellular reactive oxygen species (ROS) generation and mitochondrial damage. Conversely, cells with increased IDPm expression become more resistant to oxidative damage with less DNA fragmentation (14). Although IDPm plays an important role in cellular defense against oxidative stress-induced damage, evidence demonstrating the protective role of IDPm against oxidative injury in MI is still lacking.

Resveratrol, a polyphenol phytoalexin (trans 3, 4',5-trihydroxystilbene), is abundantly available in a wide variety of plant species. It is present in the skin and seeds of grapes and constitutes one of major components contributing to the cardiovascular protective effects of red wine (1, 11, 24, 26). Recently, resveratrol has been shown to reduce cardiomyocyte apoptosis, attenuate ventricular arrhythmias, improve post ischemic LV function and improve long-term survival in animal models of MI (3, 4, 10, 12, 13). Our previous study also has shown that resveratrol can effectively reduce infarct size and improve LV systolic and diastolic function after MI in rats (16). However, the exact molecular mechanisms of cardioprotection of resveratrol in MI are not completely understood.

Proteomic technology now allows us to examine global alterations in protein expression in the diseased heart and can provide new insights into cellular mechanisms involved in cardiac dysfunction (17). We hypothesize that proteomic profiling that evaluates multiple interacting protein species simultaneously can facilitate the discovery of exact molecular mechanisms of cardioprotection of resveratrol. The purpose of the present study is to evaluate the effects of resveratrol on MI by proteomic analysis in rats. The candidate proteins (*i.e.*, AK1 and IDPm) identified by proteomic profiling are subsequently validated by Western blot and immunohistochemical analyses.

Materials and Methods

Animal Model

The following experimental protocols were approved by the Animal Care and Use Committee of the Buddhist Tzu Chi General Hospital, Taipei branch. Two-month-old male Sprague-Dawley rats (n = 136), each with a mass of 200-250 grams, were used for this study. Left anterior descending artery (LAD) was ligated under isoflurane anesthesia as previously described (20). The rats that survived for six hours were randomized to receive daily intraperitoneal (i.p.) injections with vehicle alone (dimethyl sulfoxide, MI group) or resveratrol 1 mg/kg/day (MI/R group) for four weeks. Sham operated rats underwent all surgical procedures except the LAD ligation (sham group). Animals in the sham group were also given the vehicle by daily i.p. injections for four weeks. Echocardiographic studies and hemodynamic studies were performed four weeks after LAD ligation as described previously (15). While still under anesthesia, the animals were killed and the hearts were removed. The LV was excised and sliced into three transverse sections from the apex to the base. The middle ring was used for histologic studies. The apex and base of the LV were stored at -80°C until further proteomic studies.

Two-Dimensional Gel Electrophoresis (2-DE) and In-Gel Digestion

Tissues from the LV of sham, MI and MI/R rats were homogenized in Tris buffer. Protein concentration was determined using the Bradford protein assay with BSA as a standard. Following the protein assay, we combined the extracts from 3 hearts in each group and ran one gel per group (23). The same amount of protein from each sample was diluted with a rehydration buffer containing 1% dithiothreitol. The samples were then rehydrated overnight on 13-cm IPG strips (pH range 3-10) at 50 V on an Ettan IPGphor II isoelectric focusing (IEF) system (GE Healthcare, Newcastle, UK). After rehydration was complete, the IPG strips were reduced and alkylated in an equilibration buffer containing 75 mM Tris-HCl, pH 8.6 buffer,

6 M urea, 2% SDS, and 30% glycerol. Second dimension sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5% polyacrylamide gel. After electrophoresis, the gels were fixed in a 40% ethanol and 10% acetic acid solution, and then stained with silver staining. The gels were then destained and scanned with a master imager (Microtek ScanMaker 9800XL, Shanghai, PRC). Gel patterns of sham, MI and MI/R groups were compared with ImageMaster 2D Platinum 6.0 analysis system (Typhoon TRIO, GE Healthcare, Newcastle, UK).

The samples were run in triplicates and the results were presented as the mean of triplicate measurements. Protein spots that showed at least a 1.5-fold alteration in density relative to the sham group (density ratio > 1.5 or < 0.67) were excised for further identification. The excised gels were digested with trypsin (Promega, WI, USA) overnight at 37°C. The resulting extracts were analyzed by mass spectrometry (MS).

Protein Identification by MS

The peptide mixture was analyzed on a 4800 Proteomics Analyzer, a matrix-assisted laser desorption ionization time of flight (MALDI TOF-TOF) MS (Applied Biosystems, Bedford, MA, USA). The peptide mixture was mixed with α-cyano-4-hydroxycinnamic acid (Agilent Technologies Co. Ltd., Santa Clara, CA, USA) and spotted onto a stainless steel MALDI sample target plate. MS and tandem MS (MS/MS) analyses of the peptides were performed. Data acquisition and spectral processing were carried out using Analyst and BioAnalyst™ software from Applied Biosystems. Spectra were processed and analyzed using the MASCOT software (Matrix Science, London, UK) to search for the peptide mass fingerprints and MS/MS data in the Swissport database. The protein identifications were considered to be confident when the protein score of the hit exceeded the threshold significance score of 58 ($P < 0.05$).

Validation of the Identified Proteins by Western Blot Analysis

Equal amounts of protein were loaded and separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was incubated with the first antibodies against AK1 and IDPm (Santa Cruz, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Subsequently, the membrane was incubated with the second antibody conjugated with horseradish peroxidase (HRP) for 1 h. Band densities were detected by ECL chemiluminescence (Amersham Biosciences, Buchs, Switzerland) as described by the manufacturer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA, USA) was used as an internal control. The images were scanned with a master imager (Microtek ScanMaker 9800XL, Shanghai, PRC) and semi-aquatinted with Photoshop 7.0 (Adobe).

Immunohistochemical Analysis

In order to investigate the spatial distribution of AK1 and IDPm, immunohistochemical staining was performed on the LV. Paraffin-embedded sections, 5 μm thick, were mounted on slides, deparaffinized and rehydrated. Immunohistochemical analysis was performed after heat-mediated antigen retrieval. Hydrogen peroxide (3% in distillate water) was used to block endogenous peroxidase activity. Cardiac sections were incubated with the primary antibody against AK1 or IDPm for 2 h at room temperature, followed by poly-HRP antimouse antibody for 30 mins. As the negative control, primary antibodies were omitted. Peroxidase activity was visualized using 0.5 mg/mL 3, 3'-diaminobenzidine (DAB, brown color) with 0.05% H_2O_2 as the activator. The sections were counterstained with hematoxylin and examined with a light microscopy.

Statistical Analysis

For statistical analysis of the images of the Western blots, the statistical software program SPSS 12.0 was used. Statistical analysis of the differences of blot densities between the 3 groups was carried by one-way analysis of variance (ANOVA). If significant differences were detected by ANOVA, a *post hoc* Bonferroni test was employed. Data were presented as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Animal Model

Nine rats in the sham group survived the four-week study period. After excluding rats with infarct sizes less than 10%, 11 rats in the MI group and 12 rats in the MI/R group survived the four-week study period.

Identification of Proteins by 2-DE and MS

Fig. 1 indicates the protein spots on the 2-DE spot patterns that significantly changed in expression levels between the sham and the MI groups and between the MI and the MI/R groups. Protein spots that changed in expression levels after MI were numbered. The spot numbers in Fig. 1 refer to the numbers in Table 1 which lists these protein spots and identifica-

Table 1. Protein spots on the 2-DE image significantly changed in expression level after MI and resveratrol treatment

Spot Number	Protein Density Sham	MI	MI/R	Protein Name	Mass (Da)	pI	Sequence Coverage	MASCOT Score
1	24	7	22	Heat-shock protein beta-6	17552	6.05	28%	156
2	56	29	83	Myosin light polypeptide 4	21385	4.96	35%	141
3	118	75	91	Adenylate kinase 1	21686	7.66	35%	175
4	98	32	93	Troponin I	24261	9.57	27%	183
5	68	20	81	Peroxiredoxin-6	24861	5.64	48%	146
6	112	73	95	Glutathione S-transferase Mu 2	25860	6.90	16%	62
7	99	29	85	SCAD	45027	8.47	36%	332
8	100	34	103	Beta-enolase	47332	7.08	7%	113
9	159	47	129	Aspartate aminotransferase	47690	9.13	32%	249
10	87	20	94	ODO2	49242	8.89	19%	87
11	157	63	158	IDPm	51399	8.88	23%	191
12	152	40	158	Desmin	53482	5.21	57%	926
13	137	54	141	UQCRC1	53511	5.57	20%	52
14	18	186	83	Myosin light polypeptide 3	22258	5.03	62%	273
15	46	95	36	Albumin	70716	6.09	24%	429

Numbers refer to the spot numbers given in Fig. 1. SCAD, short-chain specific acyl-CoA dehydrogenase; ODO2, dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex; IDPm, mitochondrial $NADP^+$-dependent isocitrate dehydrogenase; UQCRC1, ubiquinol-cytochrome-c reductase complex core protein 1.

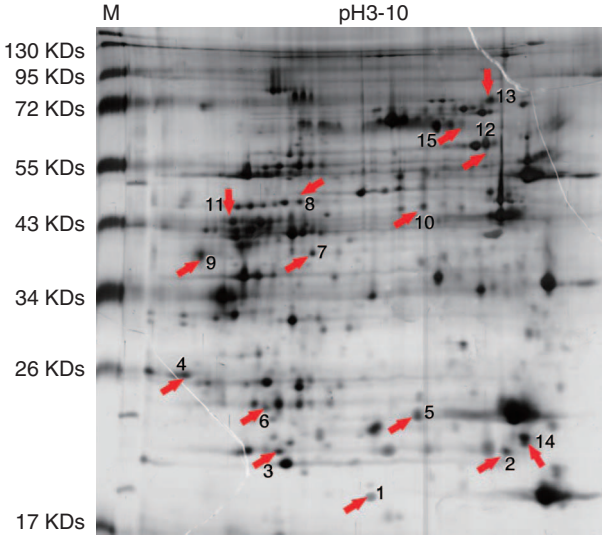

Fig. 1. Protein spots on a 2-DE gel from the MI/R rats. Numbered spots indicate protein spots displaying significant changes in expression level after MI and resveratrol treatment (density ratio > 1.5 or < 0.67).

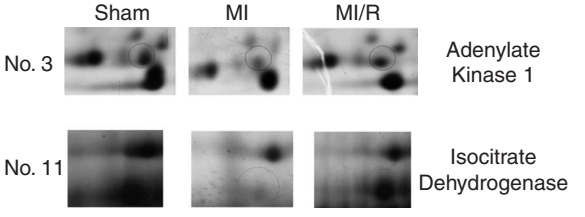

Fig. 2. Magnified 2-DE pattern of AK1 and IDPm in the sham, MI and MI/R rats. Both proteins showed downregulated expression after MI and were upregulated by resveratrol treatment.

tion. The protein density in different experimental groups, the experimental mass and isoelectric point (pI) and the MASCOT score are also listed in Table 1.

Fifteen protein spots were up- or down-regulated significantly after MI and resveratrol treatment. Of particular interest was the variation of AK1 and IDPm, which have been known to increase myocardial energetic efficiency (21) and reduce ROS mediated damage (7, 27), respectively. As shown in Fig. 2, expressions of AK1 and IDPm in the MI group was decreased compared with the sham group. These changes were attenuated by the treatment of resveratrol (Fig. 2).

Validation of the Identified Proteins by Western Blot

The findings of proteomic analysis were verified using Western blots for AK1 and IDPm. The expression levels of AK1 and IDPm were normalized by GAPDH. As shown in Fig. 3, the expression of AK1 was significantly reduced in the MI rats compared to the sham rats (65.0 ± 4.3 *vs*. 92.3 ± 3.6, $P < 0.001$). This change was significantly attenuated in the MI/R

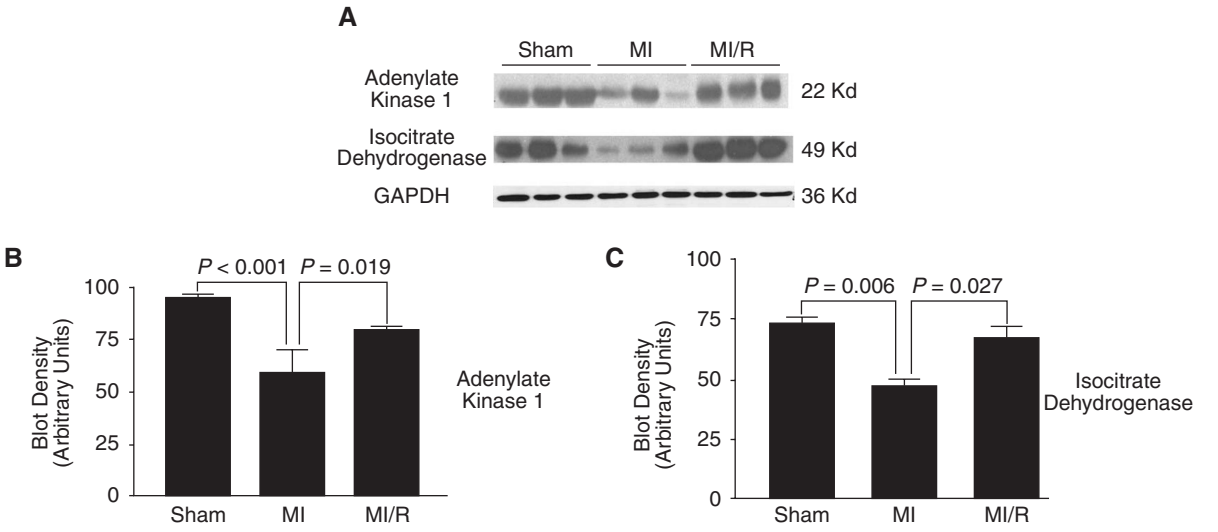

Fig. 3. Western blot analysis of AK1 and IDPm in the sham (n = 9), MI (n = 11) and MI/R (n = 12) rats. A: Re-presentative Western blot picture of AK1, IDPm and GAPDH in different groups. B and C: The expression levels of AK1 and IDPm were significantly reduced in the MI rats compared to the sham rats. The changes were significantly attenuated in the MI/R rats.

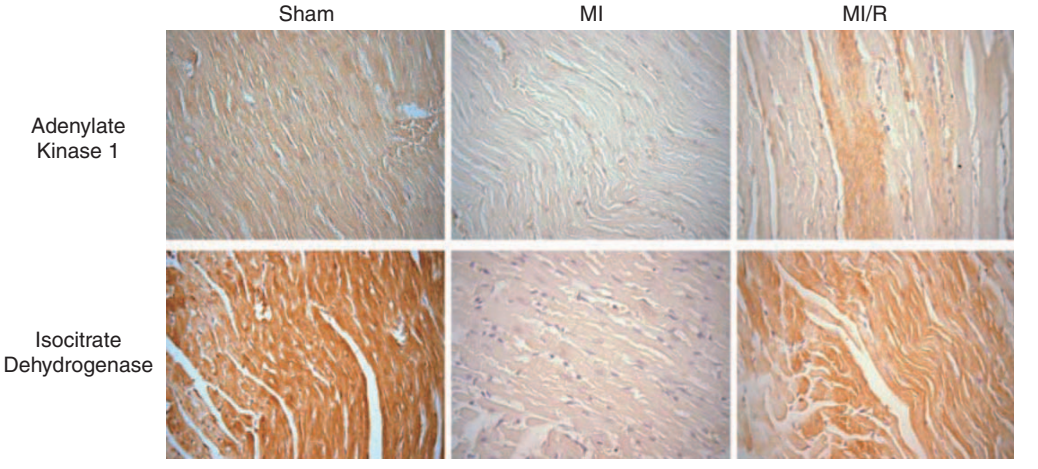

Fig. 4. Representative immunohistochemical staining for AK1 and IDPm in the sham, MI and MI/R rats. Positive stainings for AK1 and IDPm were mostly distributed in the myocardium. The intensities of AK1 and IDPm were lower in the MI rats than in the sham rats. The changes were reversed in the MI/R rats.

rats (79.5 ± 3.0, $P = 0.019$ *vs*. MI rats). The expression of IDPm was also significantly reduced in the MI rats compared to the sham rats (50.0 ± 4.3 *vs*. 74.8 ± 4.2, $P = 0.006$). This change was significantly reversed in the MI/R rats (68.9 ± 5.9, $P = 0.027$ *vs*. MI rats). There was no significant difference in the expression of AK1 or IDPm between sham rats and MI/R rats ($P = 0.064$, $P = 1.000$, respectively).

Immunohistochemical Analysis

Representative results from the immunohistochemical study are shown in Fig. 4. Immunohistochemical analysis of the LV revealed the presence of IDPm in the cytoplasm of cardiomyocytes. Endothelial cells of capillaries and fibroblasts in connective tissue were weakly stained, as previously described (14). Positive immunohistochemical staining for AK1 was also observed in the cytoplasm of cardiomyocytes. The intensities of the immunoreactions for IDPm and AK1 were both reduced in the MI group compared with that in the sham group. These changes were reversed by resveratrol.

Discussion

In our previous study, resveratrol reduced infarct size and improved LV systolic function in rats with

MI. Although we had found that reduced expression of myocardial atrial natriuretic peptide (ANP) and TGF-β1 mRNA was related to the cardioprotective effects of resveratrol (16), we hypothesized that proteomic profiling of differentially expressed proteins after MI could facilitate the discovery of other possible mechanisms. In the present study, we identify several proteins probably involved in the cardioprotective effects of resveratrol. Fifteen protein spots showed significant change in expression levels after MI and resveratrol treatment. Given the fact that resveratrol improves ventricular contractility and prevents oxidative damage after MI (9, 16, 18), we are particularly interested in the increased expression of AK1 and IDPm in the MI/R group compared with the MI group. AK1 and IDPm have been known to relate to myocardial energetic efficiency (21) and ROS mediated damage (7, 27), respectively.

In cardiomyocytes, the production of ATP primarily occurs in the mitochondria, which are separated from ATP-consuming sites in the myofibrils (8). The presence of creatine kinase, adenylate kinase and nucleoside diphosphate kinase facilitates ATP/ADP exchange between mitochondria and cytosol (7). In intact myocardium, the net adenylate kinase–catalyzed phosphotransfer rate is 10% of the total ATP turnover rate. In pacing-induced failing heart, adenylate kinase–catalyzed phosphotransfer increased and contributed 21% to the total ATP turnover (8). In myocardial and vascular tissue, AK1 is the major adenylate kinase isoform. It serves as a metabolic hub connecting other adenylate kinase isoforms, creates a continuous phosphotransfer network and mediates energy transfer between cell compartments (6). AK1 gene deletion lowers the muscle energetic efficiency, compromises the contractile force and reduces the tolerance to ischemic stress (21). In addition, AK1 deletion blunts vascular adenylate kinase phosphotransfer and precipitated inadequate coronary reflow following ischemia-reperfusion (6). In this study, the expression of AK1 was found to decrease 4 weeks after MI, which could be reversed by the treatment of resveratrol in the MI/R group. Since resveratrol increases AK1 expression, it is possible that the MI/R rats might have attained ATP more rapidly during myocardial contraction and had a higher myocardial energetic efficiency. This finding may help explain why the MI/R rats had better LV systolic function in our previous study (16).

The isocitrate dehydrogenases (IDH) may play a significant role in defending against oxidative damage. It is known that mammalian tissues contain three classes of IDH isoenzymes: IDPm, mitochondrial NAD^+-dependent IDH and cytosolic $NADP^+$-dependent IDH (15). In mitochondria, IDPm plays an important role in cellular defense against oxidative damage by supplying NADPH. NADPH is an essential reducing equivalent for the regeneration of glutathione (GSH) by glutathione reductase. Elevation of mitochondrial NADPH and GSH by IDPm in turn suppresses the oxidative stress and concomitant ROS mediated damage (27). IDPm is induced by oxidative stress. Decreased expression of IDPm markedly increases the rate of cell death, DNA fragmentation, intracellular ROS generation and mitochondrial damage with loss of intracellular ATP levels. Conversely, overproduction of IDPm protein efficiently protects the cells from ROS-mediated damage (14). In our study, the expression of IDPm decreases after MI, which can be reversed by the treatment of resveratrol. Since resveratrol increases IDPm expression, the myocardium of MI/R rats may become more resistant to oxidative damage after MI. This finding may explain why resveratrol suppressed oxidative damage in previous studies (9, 18) and why resveratrol reduced infarct size after MI in our previous studies (16).

Recent studies have shown that biomarkers appear to be useful in the diagnosis of heart failure, in risk stratification or in monitoring therapy (2). For example, levels of plasma B-type natriuretic peptides independently predict risk of death, heart failure and recurrent MI in patients with acute MI (22). However, previous molecular biologic methods usually discover deleterious upregulated biomarkers such as proteins involved in inflammation or oxidative stress (2). By comparison of gel patterns of different experimental groups, proteomic studies of MI may facilitate the discovery of novel biomarkers which are downregulated and cardioprotective in character. In our studies, the findings of downregulated AK1 and IDPm in the MI group may provide additional information regarding the pathogenesis of heart failure after MI. The variable expression levels of AK1 and IDPm in proteomic profiling, validated by Western blot and immunohistochemical analyses, suggest that proteomic analysis is an accurate and effective tool for evaluating the pathophysiology of disease.

Recent studies including ours have shown that resveratrol has a significant therapeutic effects at the dose of 1 mg/kg/day, but not at 0.1 or 0.5 mg/kg/day (10, 12, 16). We included rats treated with the dose of 1 mg/kg/day for proteomic analysis in this study. However, the dose of 5 or 22.4 mg/kg/day has also been administrated in other studies (1, 4). Further evaluation is needed to determine whether higher doses of resveratrol provide additional benefits or alter the proteomic profiling.

The present study has two other major limitations. Firstly, we did not perform AK1 or IDPm blockade during resveratrol treatment. Secondly, our study was conducted using proteins from heart tissues of rats four weeks after MI. The variations of protein

expression after MI and resveratrol treatment in our study may differ from variations in human's blood sample in the acute stage of MI. Thus, further clinical studies should be done before AK1 and IDPm can be used as biomarkers in clinical practice.

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