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ORIGINAL ARTICLE

Constitutive heat shock protein 70 interacts with α-enolase and protects cardiomyocytes against oxidative stress

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Abstract

Constitutive heat shock protein 70 (Hsc70) is a molecular chaperone that has been shown to protect cardiomyocytes against oxidative stress. However, the molecular mechanism responsible for this protection remains uncertain. To understand the mechanism associated with the myocardial protective role of Hsc70, we have embarked upon a systematic search for Hsc70-interacting proteins. Using adenosine diphosphate (ADP) affinity chromatography and mass spectrometry, we have identified α-enolase, a rate-limiting enzyme in glycolysis, as a novel Hsc70-interacting protein in the myocardium of both sham and myocardial ischemia-reperfused Sprague–Dawley rat hearts. This interaction was confirmed by co-immunoprecipitation (IP) assays in the myocardial tissues and H9c2 cardiomyocytes and protein overlay assay (POA). It was further shown that Hsc70-overexpression alleviated the H_2O_2-induced decrease of α-enolase activity and cell damage, and Hsc70 deficiency aggravated the decrease of α-enolase activity and cell damage in H_2O_2 treated H9c2 cells. Our research suggests that the protective effect of Hsc70 on the cardiomyocytes against oxidative stress is partly associated with its interaction with α-enolase.

Keywords: *Hsc70, α-enolase, myocardial ischemia-reperfusion injury, H_2O_2, affinity chromography, mass spectrometry, proteomics*

Introduction

The reperfusion of oxygenated blood following ischemia introduces a wide spectrum of injury to the myocardium. The production of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radicals and hydrogen peroxide (H_2O_2), is detected at the early stages of reperfusion. Oxidative stress caused by these free radicals has been documented to play a crucial role in the pathogenesis of myocardial ischemia-reperfusion injury.

Most cells have intrinsic defence mechanism to protect against oxidative stress/injury. Hsc70, a constitutively expressed HSP70 family member, is a cytoplasmic chaperone protein involved in the folding and trafficking of client proteins to different subcellular compartments, plays a role in the signal transduction and apoptosis process, and translocates to the nucleus after the exposure to heat shock [1,2]. Recently, a report showed that exogenous Hsc70 conferred protection to motoneurons exposed to H_2O_2 [3]. Constitutive Hsc70 could also improve cardiac preservation during oxidative insult [4]. As a pre-existed molecular chaperone in the cardiomyocytes, Hsc70 plays a more important role in protecting the myocardium against unpredictable myocardial ischemia, reperfusion or other injuries. Although the constitutively expressed Hsc70 plays a protective role against oxidative stress in the cardiomyocytes, what proteins Hsc70 could interact with, and how Hsc70 could protect against injury of myocardiocytes by these protein–protein interactions are largely unknown. So we have embarked on a systematic search for Hsc70-interacting proteins in the myocardium during

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ischemia-reperfusion injury and examined the possible effects of these protein–protein interactions.

Constitutive Hsc70 has similar structures and properties to inducible Hsp70 [5], and the two proteins contain three functional domains including the nuclear binding domain (NBD-ATPase), the substrate binding domain (SBD) and the carboxy-terminal domain (CTD), which have high affinity binding to adenosine diphosphate (ADP) [6]. In this study, we isolated Hsc70-interacting proteins in ischemia-reperfused rat hearts by ADP affinity chromatography and proteomic approach and attach importance to the interaction of Hsc70 with α-enolase and the protective effect of this interaction on cardiomyocytes against oxidative stress.

Methods

Myocardial ischemia-reperfusion model

Male Sprague–Dawley rats weighing 250–300 g were anesthetized with medetomidine, 0.5 mg/kg subcutaneously (s.c.), and ketamine 70–80 mg/kg intraperitoneally. The body temperature was monitored with a rectal probe and maintained at 37°C by a heating lamp. A cannula was inserted into the trachea and the rats were ventilated room air with a positive pressure respirator, and the heart was rapidly exteriorized through a left thoracotomy and pericardial incision. The left anterior descending coronary artery (LAD) was ligated with a 6-0 silk suture for 30 min, and reperfused for 1 h. At the end of reperfusion, the infarct size was determined by 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. The sham-operated rats underwent the same procedure, except for the ligation of the coronary artery. At least nine rats were included in each experimental group. The investigation followed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1996) and the Animal Care and Use Committee of Central South University. The study protocol was approved by the Ethics Committee of Central South University, Medical Institution Animal Care and Research Advisory Committee (Changsha, China).

Purification of Hsc70-interacting proteins by ADP-agarose affinity chromatography

After 30 min ischemia and 1 h reperfusion, the rat hearts were harvested. The left ventricular myocardial tissues were washed three times with cold PBS and homogenized with a hand-held homogenizer in cold lysis buffer A (20 mM Tris-acetate pH 7.5, 20 mM NaCl, 15 mM 2-mercapthoethanol, 3 mM $MgCl_2$, and a cocktail of protease inhibitors purchased from Roche Applied Science). The supernatants were collected by centrifugation at 100 000 rpm for 30 min at 4°C and then applied to an ADP-agarose (Sigma) column by virtue of the Hsc70 binding to ADP for affinity purification. The column was first washed with five bed volumes of lysis buffer A (see above), then with five bed volumes of wash buffer B (10 mM Tris-acetate, 150 mM NaCl, pH 7.5) and finally with two bed volumes of wash buffer C (10 mM Tris-acetate, 300 mM NaCl, pH 7.5). The Hsc70 and Hsc70-interacting proteins were eluted with elution buffer (10 mM Tris-acetate, 150 mM NaCl, 4 mM ADP, pH 7.5). The samples were then concentrated by ultrafiltration with Ultra-4 centrifugal filter devices (Amicon) and precipitated by cold dimethylketone.

Two-dimensional gel electrophoresis

The proteins were dissolved in lysis buffer (7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.5 mM EDTA, 40 mM Tris, 2% NP40, 1% Triton X-100, 5 mM PMSF, and 2% Phamarlyte) at 4°C for 1 h, and then centrifuged at 12,000 rpm for 30 min at 4°C. The supernatant was transferred to a fresh tube, and stored at −80°C until two-dimensional gel electrophoresis (2-DE). The concentration of the total proteins was measured by 2D Quantification kit (Amersham Biosciences). The 2-DE was performed to separate the Hsc70-interacting proteins from the ischemia-reperfusion and sham groups. Briefly, 650 μg of protein samples was diluted to 450 μL with a rehydration solution [7 M urea, 2 M thiourea, 0.2% DTT, 0.5% (v/v) pH 3-10 IPG buffer, and trace bromophenol blue], and applied to IPG strips (pH 3–10, length 24 cm) by 14 h rehydration at 30 V. The proteins were focussed successively for 1 h at 500 V, 1 h at 1 000 V and 8.5 h at 8 000 V for a total of 68 kVh on an IPGphor (Amersham Biosciences). Focused IPG strips were equilibrated for 15 min in a solution [6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-HCl (pH 8.8) and 1% DTT], and then for an additional 15 min in the same solution except that DTT was replaced by 2.5% iodoacetamide. After the equilibration, SDS-PAGE was done on Ettan DALT II system (Amersham Biosciences). After the SDS-PAGE, the Blue Silver staining method, a modified Neuhoff's colloidal Coomassie blue G-250 stain, was used to visualize the protein spots in the 2-DE gels [7].

In-gel protein digestion and mass spectrometry

All the interesting protein spots were excised from stained gels with a punch. Each sample was soaked sequentially with NH_4HCO_3 and NH_4HCO_3/acetonitrile, dehydrated with acetonitrile (Fisher Scientific, Loughborough, UK), and then subjected to reduction of the disulfide spots with DTT at 54°C and to alkylation with iodacetamide. The spots were washed again with NH_4HCO_3, NH_4HCO_3/acetonitrile

and acetonitrile, dried in a vacuum centrifuge (Eppendorf, Hamburg, Germany), then rehydrated in NH_4HCO_3, subjected to tryptic digestion overnight, and the peptide was gel-extracted [8].

The tryptic peptide was mixed with an α-cyano-4-hydroxycinnamic acid matrix solution. One microliter of the mixture was analyzed with a Voyager System DE-STR 4307 MALDI-TOF Mass Spectrometer (ABI) to obtain a peptide mass fingerprint (PMF). The Mascot search engine was used for rapid protein identification by peptide mass fingerprinting.

Cell culture and treatment

Rat H9c2 cardiomyocytes were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences and routinely grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. H_2O_2 was used in the medium 0.5 mM for 2 h [9].

Plasmid construction and cell transfection

A recombinant plasmid pET11α/rHsc70 carrying the full-length rat Hsc70 was a generous gift from Professor Thomas Ratajczak at the Department of Endocrinology & Diabetes, Sir Charles Gairdner Hospital, Western Australia. The plasmid was digested with BamHI and XbaI, and ligated into the pcDNA3.1(-) vector (Strategene), and the insert sequence was confirmed by sequencing (Invitrogen). Hsc70-RNAi plasmid and PGCsi.U6/neo/GFP RNAi-NC plasmid as a negative RNAi control were synthesized by Shanghai GeneChem Co., Ltd. (Shanghai, China). Rat H9c2 cardiomyocytes were cultured to subconfluence and transfected with each of the expression plasmids manufactured as described above, or the empty vector without the cDNA (control) with a Lipofectamine-mediated method (Lipofectamine 2000, Invitrogen) [10].

Co-immunoprecipitation and Western blot

Co-IP was performed as previously described [11]. Rat myocardium extract and H9c2 cells were lysed at 4°C in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture), and the lysates were cleared by a brief centrifugation (12,000 rpm for 30 min). Concentrations of proteins in the supernatant were determined by the BCA assay. Before the co-IP, samples containing equal amount of proteins were pre-cleared with protein A or protein G agarose/Sepharose (Amersham Biosciences) (4°C, 3 h), and subsequently incubated with various irrelevant IgG or specific Abs (5 μg/ml) in the presence of protein A or G agarose/Sepharose beads for 2 h or overnight at 4°C with gentle shaking. After the incubation, agarose/Sepharose beads were washed extensively with PBS, and proteins were eluted by boiling in 2 × SDS sample buffer before the SDS-PAGE electrophoresis.

For Western blot, proteins in the whole-cell lysates or tissue lysates were resolved on 12% SDS-PAGE gel, and transferred to a polyvinylidene fluoride membrane. After blocking, the membrane was incubated for 2 h at 25°C with various primary Abs specific for Hsc70 or Hsp70 (Stressgen), α-enolase (Santa Cruz Biotechnology), and IgG or actin (Boster Biotech). After incubation with peroxidase-conjugated secondary Abs for 1 h at 25°C, the signals were visualized by diaminobenzidine detection (Boster Biotech) according to manufacturer's instruction.

Protein overlay assay

Protein overlay assay (POA) was performed according to the methods of Sinclair et al [12]. For Hsc70 protein overlay assay, whole tissue lysates of the rat myocardium were extracted with 50 mM PBS, pH 8.0, containing 0.1% Triton X-100, and the soluble proteins were diluted in the PBS, pH 6.5, containing 2% BSA and 0.2% Tween 20. This solution was incubated at room temperature with immunoblots of purified Hsc70 (Stressgen) that had been separated by SDS-PAGE without heating or reducing the samples. We detected α-enolase from the myocardium extract that had bound to Hsc70 with anti-α-enolase Ab as described previously. For protein overlay assay of Hsc70, purified Hsc70 was diluted to 30 μg/ml in the PBS, pH 6.5, containing 2% bovine serum albumin (BSA), 0.2% Tween 20. This solution was incubated at room temperature with blots of SDS-PAGE-separated myocadium proteins that were not heated or reduced before the electrophoresis.

Alpha-enolase activity assay

Enzymatic activity of α-enolase was determined spectrophotometrically by monitoring the reductive reaction of 2-phosphoglycerate (2-PG) to phosphoenol pyruvate (PEP) at A240 nm [13]. An assay mixture (1 mL) containing 2.2 mM 2-PG (Sigma), 10 mM $MgSO_4$, 40 mM KCl and 300 μg whole cell lysate in 50 mM Tris-HCl, pH = 7.4, was used. One unit of enzyme was defined as the amount of enzyme converting 1 μmol substrate (2-PG) into product (PEP) in 1 min at 37°C. The accumulation of PEP was measured every 30 s and monitored for 5 min.

Cell viability MTT assay

Rat H9c2 cells were cultured in a 96-well plate at 2000 cells/well. Following the experiment in each

group, cells in the wells were treated with 20 µl 3-(4, 5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (5 g/l) (Boster Biotech) and incubated for 4 h in darkness at 37°C. Afterwards, 100 µl formazan (Boster Biotech) was added to each well and maintained for 15 min. The absorbance of the solution was analyzed by spectrophotometer at 570 nm. All experiments were performed three times.

Lactate dehydrogenase (LDH) release

The cell injury was monitored by the release of lactate dehydrogenase (LDH) into the cultured medium. The supernatant of H9c2 cells was used for the activity assay with Cytotoxicity Detection Lactate Dehydrogenase kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. Optical densities resulting from LDH activity were measured in a microplate reader (Bio-Tek Instrumnents, Inc) at 440 nm.

Flow cytometric analysis

The cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in ice-cold PBS, and fixed with 70% ethanol. All the fixed cell samples were delivered to Beijing Dingguo Biotechnology to evaluate the apoptosis with Coulter Epics XL.

Statistical analysis

The data are presented as mean ± S.E.M. Statistical significance ($p < 0.05$) for each variable was estimated by analysis of variance (ANOVA) followed by a Dunnett's *post hoc* analysis.

Results

2-DE Maps of ADP-purified proteins between the sham and I/R group

Two representative 2-DE maps from pooled samples are shown in Figure 1A and B. A total of 41 protein spots between the sham and ischemia/reperfusion (I/R) heart tissues in triplicate experiments were chosen and subjected to MS analysis, and 32 protein spots identified by MS are marked with arrows in Figure 1A and B. A close-up of the region of the gels of protein spot 31 in the sham and I/R groups is shown in Figure 1C.

Identification of ADP-purified proteins by MS

A total of 32 differential proteins were successfully identified by MALDI-TOF-MS (Table I). The MALDI-TOF mass spectrometry map and database query of the spot 31 are shown in Figure 2A and B. A total

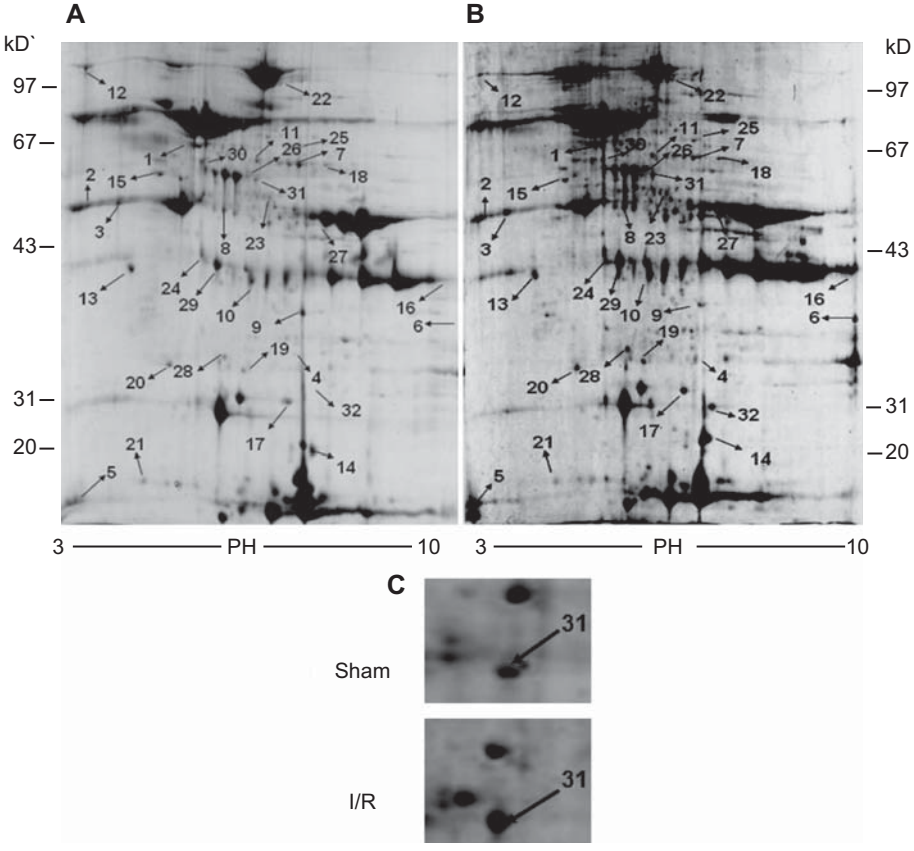

Figure 1. Representative 2-DE maps of the sham (A) and I/R group (B). The protein spots marked with arrows were 32 protein spots identified by MS. (C) Close-up of the region of gels of protein spot 31 in the sham and I/R group.

of 13 monoisotopic peaks were input into Mascot search engine to search the Swiss–Prot database, and the query showed that the protein spot 31 was α-enolase. The annotation of all the identified proteins is summarized in Table I.

Identification of Hsc70-interacting proteins by co-immunoprecipitation and Western blot

Both Hsp70 and Hsc70 can bind to ADP, so we used antibodies against Hsc70 and Hsp70 to investigate whether Hsc70 or Hsp70 was expressed in our models. Western blot showed that only Hsc70 was expressed in the myocardium after 30 min ischemia and 1 h reperfusion and in H9c2 cells exposed to H_2O_2 (0.5 mM) for 2 h (Figure 3A). The interaction of Hsc70 and α-enolase was validated by co-IP experiments using antibodies against α-enolase and Hsc70, respectively (Figure 3B and C). Both the cell lysates and heart extracts were pre-cleared by protein A/G beads before the co-IP experiments. Hsc70 was detected in the co-IP complex pulled down with the anti-α-enolase antibody; α-enolase was also detected with anti-α-enolase antibody in the co-IP complex pulled down with Hsc70 antibody. Both panel B and C showed that antibodies against α-enolase or Hsc70 were able to immunoprecipitate their binding counterparts from the heart extracts and cell lysates, but the same samples incubated with the non-specific rabbit IgG could not. This two way co-IP assay suggested an interaction between α-enolase and Hsc70.

POA of Hsc70 and α-enolase

To ensure the interaction of Hsc70 and α-enolase, we performed *in vitro* POA. Overlay assay of Hsc70 revealed purified Hsc70 with Hsc70-antibody in the α-enolase band site (48 kDa) (Figure 4A). α-enolase overlay assay revealed α-enolase by anti-α-enolase antibody in the band sites of Hsc70 (Figure 4B), further suggesting that α-enolase interacted with Hsc70 *in vitro*.

Table I. Proteins after ADP affinity chromatography in sham and I/R group identified by MALDI-TOF-MS.

Spot no.	Protein name	Accession no.	Mass (Da)	p*I*	Sequence coverage	Scores	Principal function
1	Heat shock protein 60kDa	P63039	61088	5.91	58%	231	chaperone
2	Creatine kinase	P09605	47811	8.76	50%	217	metabolic enzyme
3	Actin,aortic smooth muscle	P62738	42334	5.23	42%	108	structural protein
4	Phosphoglycerate mutase 1	P25113	28928	6.67	23%	59	metabolic enzyme
5	Nucleoside diphosphate Kinase B	P19804	17386	6.92	90%	170	signal transduction
6	Glyceraldehyde-3-phosphate dehydrogenase	P04797	36090	8.14	45%	155	metabolic enzyme
7	Dihydrolipoyl dehydrogenase	Q6P6R2	54574	7.96	58%	123	metabolic enzyme
8	Dihydrolipoyllysine-residue uccinyltransferase component of 2-oxoglutarate dehydrogenase complex	Q01205	49236	8.89	43%	128	metabolic enzyme
9	Electron transfer flavoprotein	P13803	35272	8.62	72%	239	metabolic enzyme
10	Pyridoxal kinase	035331	35114	6.32	54%	164	metabolic enzyme
11	T-complex protein 1	Q5XIM9	57764	6.01	51%	200	chaperone
12	2-oxoglutarate dihydrogenase	Q5XI78	117419	6.3	44%	374	metabolic enzyme
13	Tropomyosin	Q63608	32718	4.69	48%	132	structural protein
14	Alpha-crystallin B chain	P23928	20076	6.76	66%	180	chaperone
15	ATP synthase	P10719	56318	5.19	61%	238	metabolic enzyme
16	Malate dehydrogenase, cytoplasmic	088989	36631	6.16	33%	109	metabolic enzyme
17	Glutathione peroxidase 1	P04041	22463	7.7	71%	192	metabolic enzyme
18	Long-chain specific acyl-CoA dehydrogenase	P15650	48242	7.63	48%	201	metabolic enzyme
19	Peroxiredoxin-3	Q9ZOV6	28563	7.14	37%	115	metabolic enzyme
20	Myosin light chain 3	P**I**6409	22356	5.03	45%	82	structural protein
21	Myosin regulatory light chain 2	P08733	18868	4.86	33%	62	structural protein
22	Glycogen phosphorylase	P09812	96854	6.24	19%	51	metabolic enzyme
23	C-terminal-binding protein 1	Q9Z2F5	47055	6.17	44%	186	transcription regulatory
24	N-acetyl-D-glucosamine kinase	P81799	37458	5.55	38%	176	metabolic enzyme
25	Stress-induced-phosphoprotein 1	035814	63158	6.4	20%	107	signal transduction
26	Aldehyde dehydrogenase, mitochondrial	P11884	56966	6.63	40%	168	metabolic enzyme
27	Aspartate aminotransferase	P13221	46628	6.73	40%	174	metabolic enzyme
28	Peroxiredoxin-6	035224	24860	5.64	57%	143	metabolic enzyme
29	L-lactate dehydrogenase B chain	P42123	36874	5.7	65%	294	metabolic enzyme
30	Vitamin D-binding protein	P04276	55106	5.65	38%	128	transport
31	Alpha-enolase	P04764	47440	6.16	51%	180	metabolic enzyme
32	Adenine phosphoribosyltransferase	P36972	19761	6.17	58%	131	metabolic enzyme

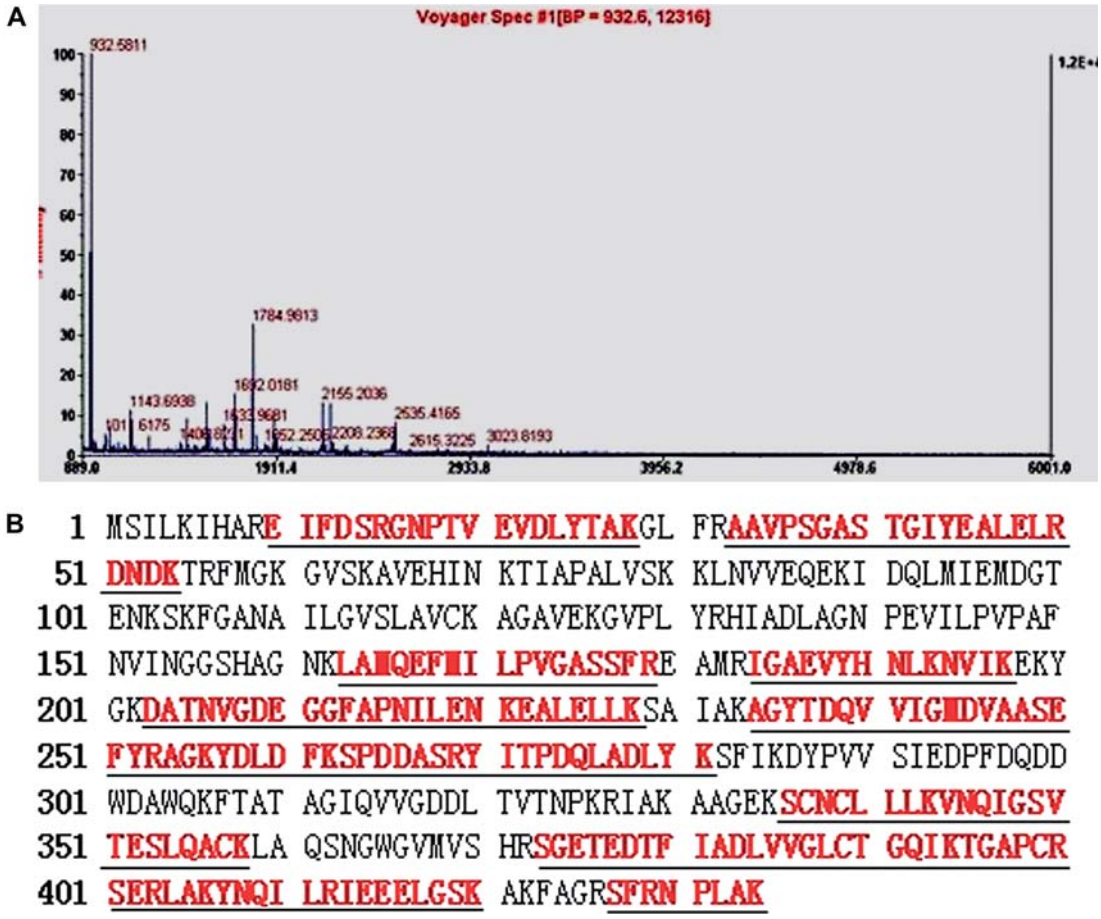

Figure 2. MALDI-TOF-MS analysis of protein spot 31. (A) MALDI-TOF-MS mass spectrum of spot 31 identified as α-enolase according to the matched peaks. (B) Protein sequence of α-enolase was shown, and matched peptides were underlined.

Effect of Hsc70-overexpression and knock-down on α-enolase enzymatic activity in H_2O_2-treated cardiomyocytes

To determine the association of Hsc70-upregulation or downregulation in H9c2 cell line with α-enolase activity, H9c2 cells were transiently transfected with pcDNA3.1(−) or pcDNA3.1(−) -Hsc70, control siRNA, and Hsc70-siRNA plasmid, respectively. Hsc70 expression was determined by Western blot, with actin as the loading control. Hybridization signals were quantified and normalized to actin signals and presented as the fold increase or decrease over the respective controls. Introduction of Hsc70 into H9c2 cells significantly increased Hsc70 expression ($p < 0.05$, Figure 5A), whereas introduction of Hsc70-siRNA into H9c2 cells significantly decreased Hsc70 expression ($p < 0.05$, Figure 5B).

The results showed that α-enolase activity decreased after the H_2O_2 exposure (0.5 mM) ($p < 0.05$, Figure 5C and D). Hsc70-overexpression alleviated the decrease of α-enolase activity after the H_2O_2 treatment as compared with the neo group. Hsc70 deficiency aggravated the decrease of α-enolase activity after the H_2O_2 treatment as compared with the control siRNA group ($p < 0.05$, Figure 5D).

Effect of Hsc70-overexpression and knock-down on H_2O_2-induced damage in the myocardiocytes

To evaluate whether the protective effect of Hsc70 is related to its interaction with α-enolase, Hsc70-overexpression and deficiency were induced as above in H_2O_2-treated cardiomyocytes. Cell viability and LDH leakage was examined. MTT assay showed that overexpression of Hsc70 diminished the loss of cell viability induced by H_2O_2 as compared with the neo group ($p < 0.05$, Figure 6A). Hsc70-deficiency resulted in significant decrease of cell viability in H_2O_2-treated cells ($p < 0.05$, Figure 6B). LDH activity assay showed that overexpression of Hsc70 in the cardiomyocytes significantly reduced H_2O_2-induced LDH release ($p < 0.05$, Figure 6C). The cardiomyocytes transfected Hsc70-siRNA significantly increased the H_2O_2-induced LDH release ($p < 0.05$, Figure 6D).

We also determined the effect of Hsc70-overexpression and deficiency on the apoptosis of cardiomyocytes induced by H_2O_2. Flow cytometry revealed that overexpression of Hsc70 significantly inhibited the apoptosis induced by H_2O_2 as compared with the neo group ($p < 0.05$, Figure 6E). The Hsc70-deficiency significantly increased the H_2O_2-induced apoptosis ($p < 0.05$, Figure 6F).

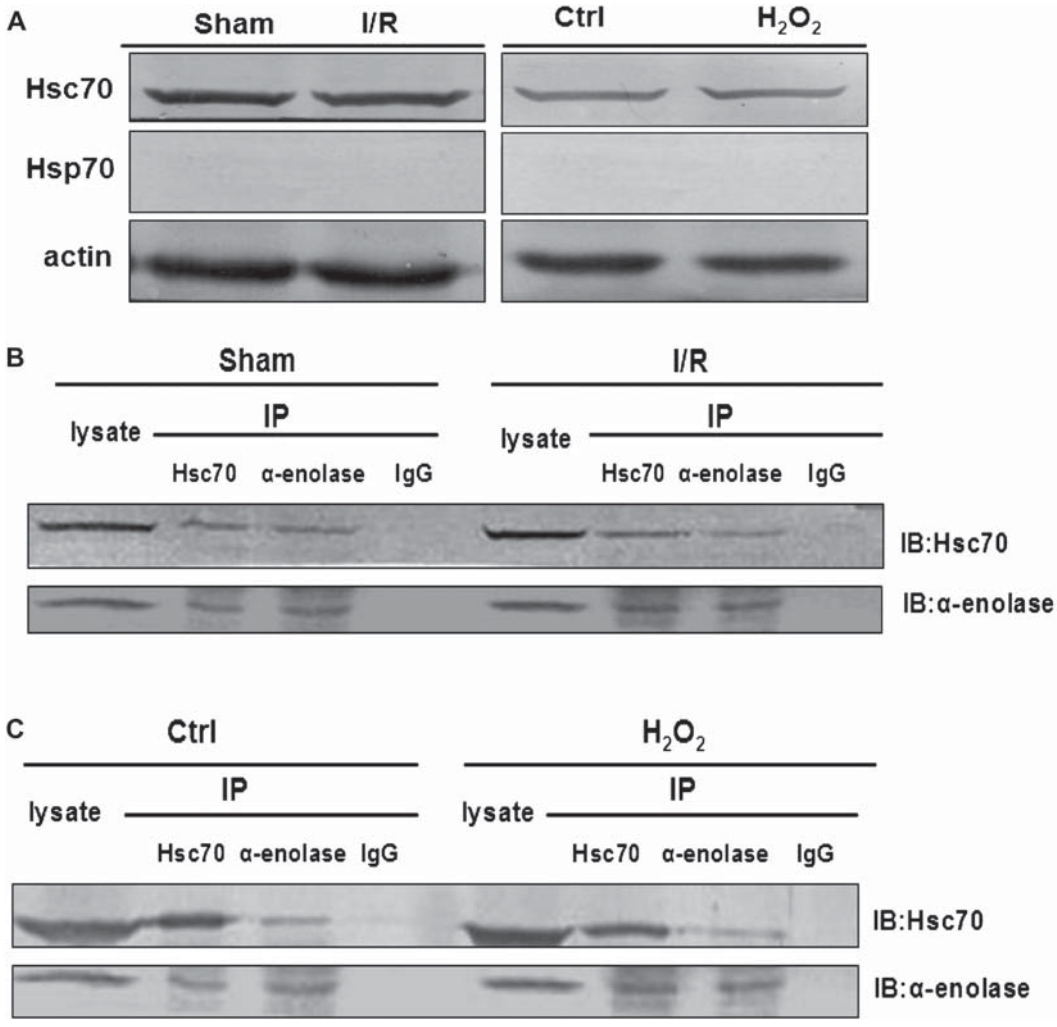

Figure 3. Confirmation of interaction between Hsc70 and α-enolase by co-immunoprecipitation (co-IP) in the myocardium and cardiomyocytes. (A) Expression of Hsc70 and Hsp70 by Western blot. In the myocardium either in sham and I/R group, or H9c2 cells in the control and H_2O_2-treated group, a high level of constitutive Hsc70 but not the inducible isoform Hsp70 was seen. (B) Co-IP of Hsc70 and α-enolase from the sham and I/R heart tissues. Preimmunoprecipitated sham or I/R heart homogenate was used as positive control (lysate), and immunoprecipitate with non-specific rabbit IgG was used as negative control (IgG). (C) Co-IP of Hsc70 and α-enolase from the control and H_2O_2-treated H9c2 cells. Preimmunoprecipitated control or H_2O_2-treated cell lysates were used as positive control (lysate), and immunoprecipitated with non-specific rabbit IgG was used as negative control (IgG). Blots are representative of three independent experiments with similar results, n = 3.

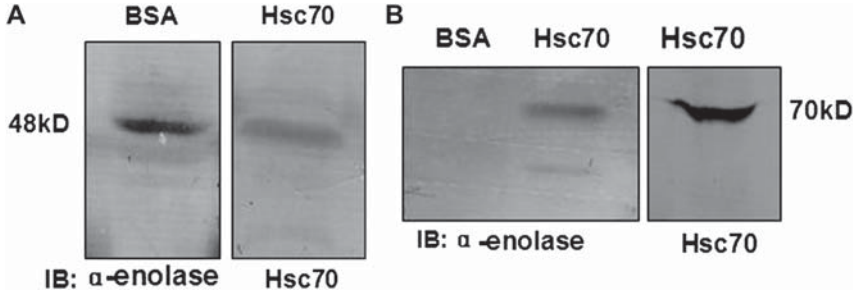

Figure 4. Protein overlay assays of Hsc70 and α-enolase. (A) Overlay analysis of Hsc70. Un-heated and un-reduced rat myocardium extracts was electrophoresed and blotted onto the PVDF membrane. The blots were incubated with purified Hsc70 at room temperature in vitro. The immuno-activity of Hsc70 was detected with anti-Hsc70 antibody (right panel). The α-enolase within the extracts was detected with anti-α-enolase antibody (left panel). (B) Overlay analysis of α-enolase. Un-heated and un-reduced purified Hsc70 was electrophoresed and blotted onto the PVDF membrane as in A, then blots were incubated with the myocardial extracts, and the immuno activity of α-enolase was detected with anti-α-enolase antibody (left panel). Hsc70 was detected with anti-Hsc70 antibody (right panel). The molecular weight of Hsc70 or α-enolase is indicated at the right. Blots are representative of three independent experiments with similar results.

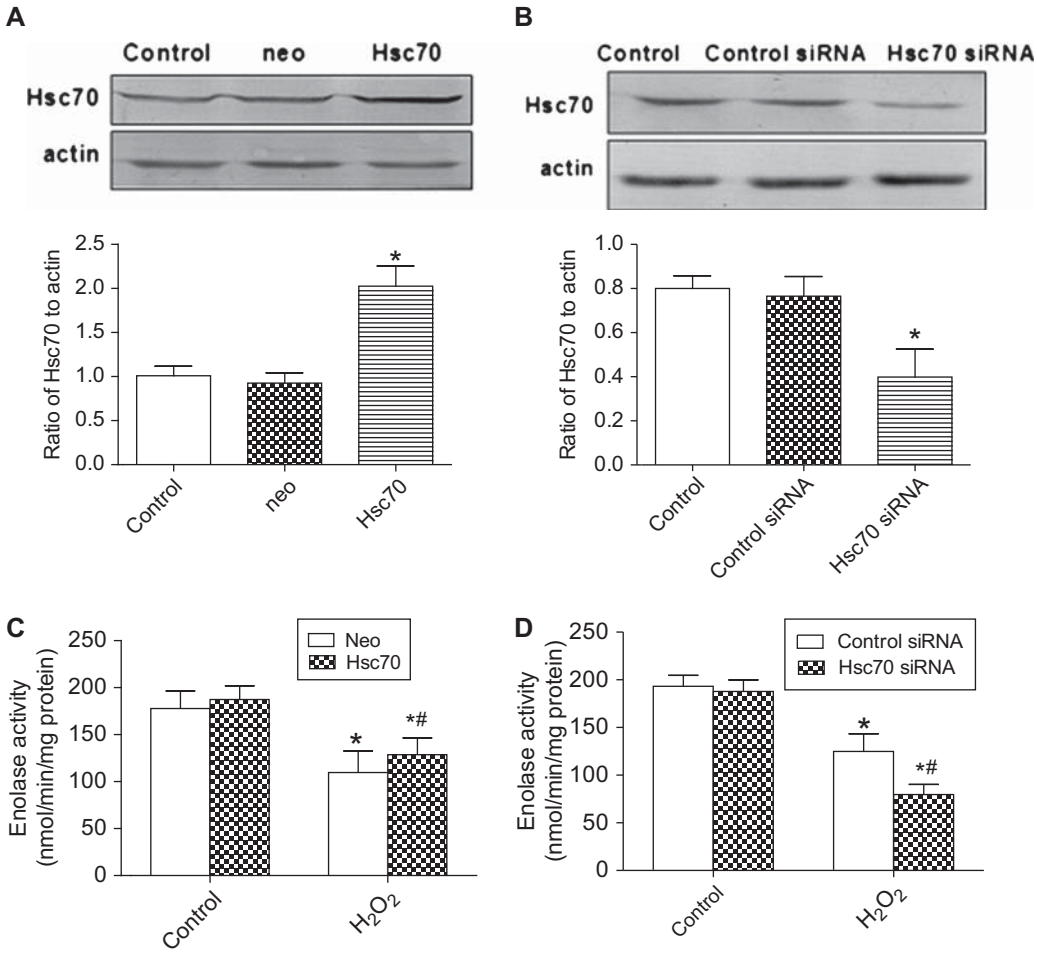

Figure 5. Effect of Hsc70-overexpression or deficiency on α-enolase enzymatic activity in H_2O_2-treated cardiomyocytes. (A) Western blot showing the expression level of Hsc70 in pcDNA3.1(-)-Hsc70 transiently transfected H9c2 cells. (B) Western blot of Hsc70 expression in Hsc70-siRNA transiently transfected H9c2 cells. (C) α-enolase activity assay in H_2O_2-treated Hsc70-overexpressed H9c2 cells. (D) α-enolase activity assay in H_2O_2-treated Hsc70 deficiency H9c2 cells by Hsc70 siRNA. Mean ± S.E. M., n = 5, $^{\star}p < 0.05$ *vs*. the control group, $^{\#}p < 0.05$ *vs*. the neo or control siRNA + H_2O_2 group.

Discussion

In this study, we have identified 32 possible Hsc70-interacting proteins and confirmed that α-enolase is a new interacting protein in the myocardium and H9c2 cells. Our findings demonstrate that Hsc70 can regulate the activity of α-enolase and protect against oxidative stress in H9c2 cells.

As summarized in Table I, most ADP purified proteins identified by MS were metabolic enzymes (such as ATP synthase, glycolytic enzymes), structural proteins (such as actin) and chaperones (such as Hsp60). Thus, the table presented a snapshot of Hsc70 engaged in not only in house-keeping, but also in metabolism. The interaction with glycolytic enzymes was previously unreported for Hsc70 but might be important to guarantee a higher production of ATP under myocardial ischemia and oxidative stress.

The enzyme α-enolase (2-phospho-D-glycerate hydrolyase), is a rate-limiting enzyme in the glycolytic pathway which catalyzes the production of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high energy intermediates in the process of ATP production [14]. α-enolase reaction occupies a key position in the metabolic pathway of fermentation in general and the glycolytic pathway, in particular, and hence this enzyme is ubiquitously present in abundance in the biological world.

It is shown that α-enolase participates in the maintenance of intracellular ATP levels in the cardiomyocytes exposed to ischemic hypoxia, which is closely related to cell survival [15]. In the working perfused heart, the control of glucose utilization in the pathway from 3-phosphoglycerate to pyruvate is shared by α-enolase and pyruvate kinase [16]. As shown in the report, the decrease in α-enolase expression leads to the suppression of ATP production in the heart ischemia, and partial ATP depletion could induce Fas and caspase mediated apoptosis in Madin–Darby canine kidney cells [17,18], while complete ATP deletion blocks Fas-induced apoptosis [19] and leads to necrosis mediated by poly (ADP-ribose) polymerase in the fibroblast cells [20]. Some investigators found that blocked α-enolase activity by anti-enolase

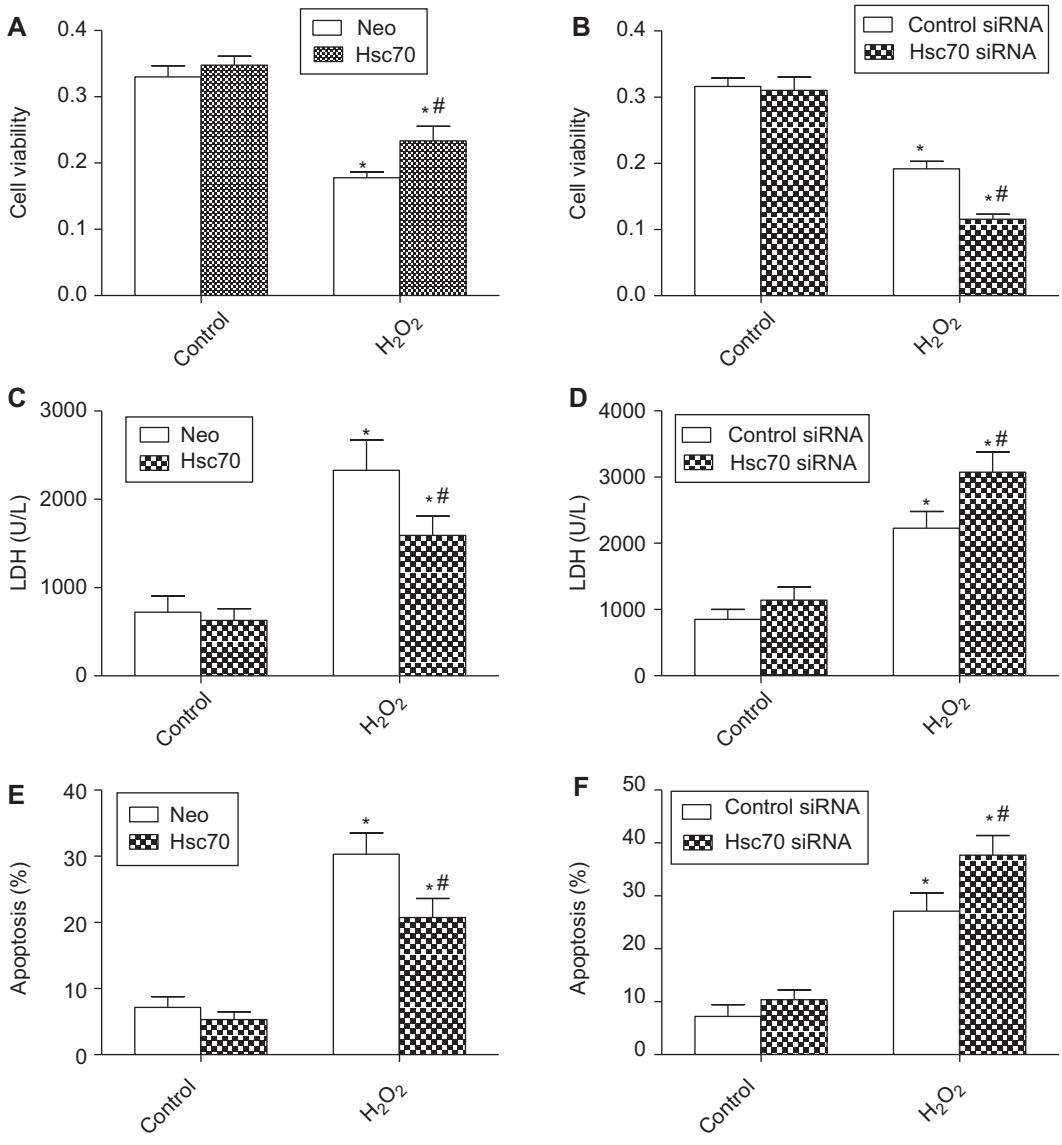

Figure 6. Effect of Hsc70-overexpression and deficiency on cell damage in H_2O_2-treated myocardiocytes. MTT assay, LDH activity assay and flow cytometry were used in H_2O_2-treated Hsc70-overexpressed or deficient H9c2 cells. (A) MTT assay in Hsc70-overexpressed group. (B) MTT assay in Hsc70-siRNA group. (C) LDH leakage in Hsc70-overexpressed group. (D) LDH leakage in Hsc70-siRNA group. (E) Apoptosis in Hsc70-overexpressed group. (F) Apoptosis in Hsc70-siRNA group. Mean ± S.E. M., n = 5; $^{*}p < 0.05$ *vs*. the control group, $^{\#}p < 0.05$ *vs*. the neo or control siRNA + H_2O_2 group.

antibody decreased the viability of retinal cells by induction of apoptosis [21]. These findings suggest that α-enolase is important for cell survival and maintenance of functions during the myocardial ischemia or ischemia-reperfusion injury. Recently, the accumulating evidence makes clear that α-enolase is multifunctional: a heat shock protein [22], a transcriptional repressor of the c-myc proto-oncogene [23], binding to the cytoskeleton and chromatin structure, a surface receptor for the binding protein of plasminogen [24], etc.

We suppose that Hsc70 interacts with glycolytic enzyme α-enolase and potentially regulates the activity and protects the functions of α-enolase in ischemia-reperfused myocardium and H_2O_2-exposed cardiomyocytes. In this study, we have confirmed that Hsc70 interacted with α-enolase through co-IP and POA. In addition, α-enolase activity assay of the proteins precipitated by anti-α-enolase antibody or anti-Hsc70 antibody from the myocardial tissue, and H9c2 cells also confirmed this interaction (data not shown). The activity of α-enolase in the cell lysate of H9c2 cells was decreased after the H_2O_2 exposure, and the decrease of α-enolase activity appeared to relate to the aggravation of cell damage as shown by cell survival, LDH release and apoptosis in the cardiomyocytes, indicating the cell damage might be associated with the inactivation of α-enolase and resultant changes in energy metabolism. These results dovetailed with a previous report that arsenite induced alpha-enolase carbonylation partially reduced the enzyme activity, leading to a decrease of

glycolysis and initiation of cell death [25]. We also found that overexpression of Hsc70 significantly alleviated the decrease of α-enolase activity and cell damage as shown by LDH release, cell apoptosis and cell survival rate in H9c2 cells after the H_2O_2 exposure. Whereas the down-regulation of Hsc70-expression by siRNA aggravated the decrease of α-enolase activity and cell damage induced by H_2O_2. These findings demonstrated that myocardial protective role of Hsc70 was partly associated with its interaction with α-enolase and subsequent activity regulation of α-enolase, and provided new clues and insight for the research on endogenous myocardial protection of heat shock protein.

Here are some limitations of this study. That Hsc70 regulated α-enolase activity and protected against oxidative stress was studied only in H9c2 cells. Although the protective role was demonstrated in H9c2 cells, this may not represent the fact in rat models, and we need to test in rat models using α-enolase inhibitors/activators. Unfortunately, no commercial inhibitors/activators of α-enolase and α-enolase transgenic or knockout animals are available, so we can't experiment in an *in vivo* rat ischemia-reperfusion model. Although we were able to isolate and identify a broad set of potentially novel Hsc70-interacting proteins, we still believe that there might be other Hsc70-interacting proteins we failed to detect due to low cellular abundance or instability. For technical reasons (difficulties in separating ischemic and non-ischemic parts of the heart), it was impossible for us to access the accurate Hsc70 targets of the two parts. We may not exclude some of the effect of medetomidine and ketamine in the myocardium. We did not observe significant changes of Hsc70 targets in the sham and I/R group, so we assumed medetomidine and ketamine did not affect our findings.

Notwithstanding these limitations, this study does suggest Hsc70 may play a critical role in glycolysis pathway and energy metabolism, which needs further identification.

Conclusions

In summary, Hsc70 can interact with α-enolase and protect against oxidative stress in H9c2 cells. These data point to new aspects of Hsc70 cardioprotective roles.

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Declaration of interest

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